SUGAR SENSING AND REGULATION OF CONIDIATION IN *NEUROSPORA CRASSA*

A Dissertation

by

XIN XIE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2003

Major Subject: Genetics

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Approved as to style and content by:

Daniel J. Ebbole (Chair of Committee)	Clint W. Magill (Member)
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ABSTRACT

Sugar Sensing and Regulation of Conidiation in

Neurospora crassa. (August 2003)

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The orange bread mold *Neurospora crassa* is a useful model for the study of filamentous fungi. One of the asexual reproduction cycles in *N. crassa*, macroconidiation, can be induced by several environmental cues, including glucose starvation. The *rco-3* gene is a regulator of sugar transport and macroconidiation in *N. crassa* and was proposed to encode a sugar sensor (Madi *et al*., 1997). To identify genes that are functionally related to RCO-3, three distinct suppressors of the sorbose resistance phenotype of *rco-3* were isolated and characterized. The *dgr-1* mutant phenotypically resembles *rco-3* and may be part of the *rco-3* signaling pathway. Epistatic relationship among *rco-3*, *dgr-1* and the suppressors were carried out by analyzing *rco-3*; *dgr-1* and *sup*; *dgr-1* double mutants. These analyses indicate that *rco-3* is epistatic to *dgr-1*.

A cDNA microarray containing 1363 *N. crassa* genes was generated to examine the transcriptional response of wild type cells grown in the presence of glucose or starved for glucose for two hours. Comparing *N. crassa* profiling data with the published diauxic shift data from *S. cerevisiae* (DeRisi *et al.,* 1997) revealed that *S. cerevisiae* and

N. crassa share a similar, but not identical, transcriptional response pattern for genes belonging to central carbon metabolism. The microarray results indicate that *N. crassa* utilizes glucose through fermentation and respiration simultaneously in aerobic culture, a finding that is consistent with previous measurements of ethanol production and enzyme activities in *N. crassa*. The same microarray was used to examine the transcriptional response to glucose status in *rco-3* and *dgr-1* mutants. The two mutants display similar expression patterns for most of the genes on the microarray supporting a close functional relationship between them. In addition, I identified a high affinity glucose transport gene in *N. crassa*, whose transcription is under the control of glucose, *rco-3* and *dgr-1*.

DEDICATION

This dissertation is dedicated to my parents, Hongming Xie and Liuhe Liu, for their love and support throughout my life. They have been wonderful examples of how to work hard and be successful parents.

谨将此文献给我的父亲-谢鸿明，母亲-刘六合，感谢他们多年来对我的关心, 爱护和支持。

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I would like to thank Dr. Ebbole for taking me as a graduate student and being patient with me. I would also like to thank Drs. Clint Magill, Marian Beremand and Deborah Bell-Pedersen for serving as committee member and giving helpful criticism. Finally, I want to thank my wonderful colleagues in Dr. Ebbole's laboratory and friends that have had an impact on my life.

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CHAPTER I

INTRODUCTION

The orange bread mold *Neurospora crassa* is well known as a useful model for the study of eukaryotic genetics generally and filamentous fungi in particular. It grows through and over the substrate, forming the branching, thread-like cells called hyphae that acquire nutrients through extracellular digestion of complex molecules and absorption of simple molecules. Under certain environmental conditions, such as nutrient deprivation, alteration in the CO_2 levels, desiccation and blue light, the fungus enters its asexual sporulation cycle, producing macroconidia (multinucleate) on specialized hyphae (conidiophore) and microconidia (uninucleate). My research is focused on environmental signals that induce macroconidiation. I will refer to this process as conidiation throughout. Upon induction, *N. crassa* hyphae give rise to aerial branches that grow away from the substrate. Within 4 hours of induction, these branches switch from hyphal elongation to budding growth. Budding produces proconidial chains with subtle interconidial constrictions, which are called "minor constriction chains" and represent the first stage of conidiophore formation. As budding continues, bud junctions narrow and form "major constriction chains" that are unable to revert to vegetative hyphal growth. The final step, septation, occurs 12 hours after induction and mature spores are found 4 hours later (Figure 1.1) (Springer and Yanofsky, 1989; Springer,

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This dissertation follows the style and format of *Molecular Microbiology*.

1993; Ebbole, 1996).

Several morphological mutants of *N. crassa* that are blocked in specific stages of conidiation have been isolated and characterized (Figure 1.1) (Matsuyama *et al.,* 1974; Perkins *et al.,* 1982; Springer and Yanofsky, 1989). Two mutants, *acon-2* (*aconidiate-2*) and *fld* (*fluffyoid*) produce aerial hyphae but are unable to produce minor constriction chains. Both mutants were proposed to sense nutrient levels to determine the switch from hyphal elongation to budding growth (Springer and Yanofsky, 1989). *fl* (*fluffy*) and *acon-3* (*aconidiate-3*) make minor constriction chains but not major constriction chains (Matsuyama *et al.,* 1974). However, *fl* mutants are well known for their elevated fertility as the female partner in sexual crosses while *acon-3* mutants are female sterile. The *fl* gene has been cloned and it encodes a Gal4p-type C6 zinc cluster protein with most sequence similarity to the *N. crassa* NIT4 protein that regulates genes required for nitrate utilization (Bailey and Ebbole, 1998). *fl* is transiently induced prior to budding growth and acts as a developmentally regulated transcription factor required for conidiophore morphogenesis (Bailey and Ebbole, 1998). The *csp-1* (*conidial-separation-1*) and *csp-2* (*conidial-separation-2*) mutants form conidiophores that do not mature to release free conidia. The *gran* (*granular*) mutant produces multiple (three to ten) buds while wild type generates only one bud. The *tng* (*tangerine*) mutant is unable to arrest growth or initiate new budding and generates gigantic macroconidia. *eas* (*easily wettable*) conidia lack the hydrophobic protein coat and are easily wettable (Perkins *et al.,* 1982; Springer and Yanofsky, 1989; Ebbole, 1996).

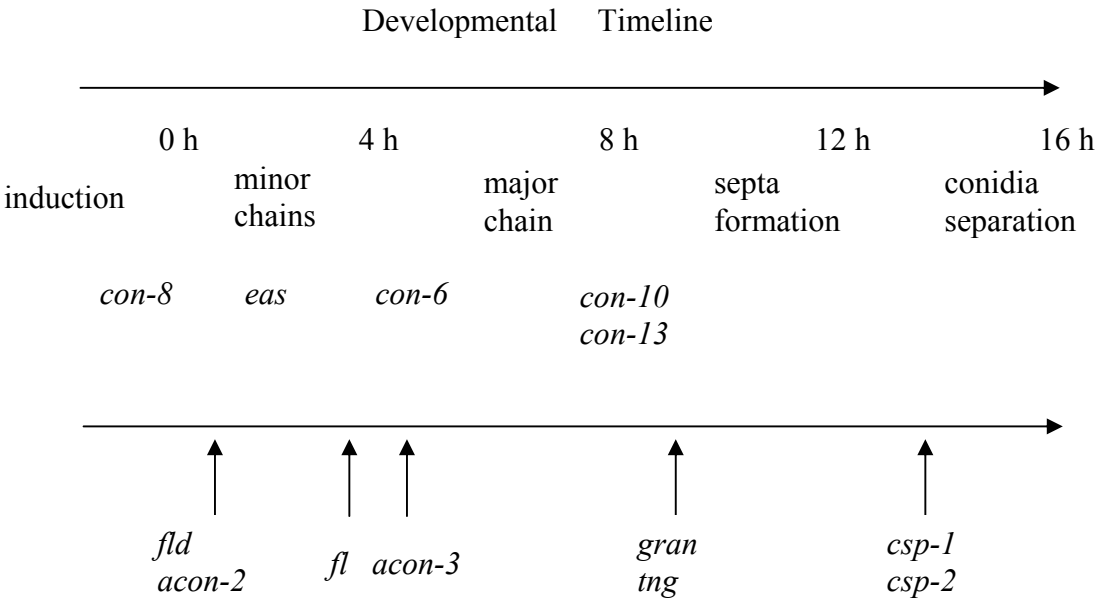


Figure 1.1 Timeline of conidiophore formation, gene expression and stages at which mutants affect morphologies in *N. crassa* (Ebbole, 1996).

A set of conidiation-specific (*con*) genes have been characterized and cloned in *N. crassa* (Figure 1.1) (Berlin and Yanofsky, 1985; Robert *et al.,* 1988; Sachs and Yanofsky, 1991). In general, *con* genes are not expressed or are expressed at low levels in the early developmental mutants (e.g. *fl*, *acon-2*, and *acon-3*) (Robert *et al.,* 1988; Robert and Yanofsky, 1989). The *con* genes can be disrupted without affecting any of the three sporulation pathways (Springer and Yanofsky, 1992). One of them, *con-10* encodes a small stress protein that is induced at the time of major constriction chain formation. It is regulated by light, the circadian clock, nitrogen or carbon starvation and heat shock (Lauter and Yanofsky, 1993; Lee and Ebbole, 1998a; Lee and Ebbole, 1998b). Mutagenesis experiments have been performed to obtain mutants that have altered regulation of *con-10* (Madi *et al.,* 1994). Two of the genes discovered by this approach have been cloned. *rco-1* (regulation of conidiation) is thought to be a cell-type specific repressor of *con-10* expression and with greatest sequence similarity to *Tup1* of *S. cerevisiae*, which is a general transcriptional repressor of genes involved in a variety of processes (Yamashiro *et al.,* 1996). The null *rco-1* mutant is blocked at the conidial separation stage, similar to *csp* mutants. But it is also pleiotropic; it has slower growth than wild type and is female sterile. The *rco-3* mutant can conidiate in submerged culture without nutrient limitation, and the gene exhibits sequence similarity to the glucose transporter gene superfamily of *S. cerevisiae* (Madi *et al.,* 1997). The mutant is 2-deoxyglucose (2-DG) resistant, sorbose resistant, and altered in glucose repression and glucose transport activities (Madi *et al.,* 1997). Therefore *rco-3* may provide a link between glucose transport, metabolism and conidiation in *N. crassa*.

Two glucose transport systems have been characterized in *N. crassa* (Scarborough, 1970; Schneider and Wiley, 1971). One is a constitutive low-affinity transport system with a Km similar to the *S. cerevisiae* low-affinity glucose transport system (Km=25 mM). This system can transport glucose, 3-O-methyl glucose, and L-sorbose. The other is a glucose-repressible high-affinity transport system, which transports glucose, 3-O-methyl glucose, L-sorbose, galactose, mannose and 2-deoxyglucose with a Km around 0.04 mM (Crocken and Tatum, 1967; Schneider and Wiley, 1971). In *S. cerevisiae*, it has been shown that glucose (hexose) transporters can transport glucose and fructose as well as other sugars (Coons *et al.,* 1995). In *N. crassa*, there is a separate glucose-repressible fructose uptake system with a Km of 0.4 mM, and L-sorbose competitively inhibits fructose uptake (Rand and Tatum, 1980).

Glucose is the primary carbon and energy source for the cells of most organisms. The first and limiting step of glucose metabolism is transport across the plasma membrane. *S. cerevisiae* has 20 genes (*hxt1 to hxt17, gal2, snf3 and rgt2*) that encode proteins similar to glucose (hexose) transporters (Bisson *et al.,* 1993; Kruckeberg, 1996; Boles and Hollenberg, 1997). Among twenty members of the *hxt* gene family, ten genes (*hxt8* to *hxt17*) encode proteins that are either unable to transport glucose or are not expressed under the conditions tested and are not thought to play an important role in glucose transport (Özcan *et al.,* 1998). Seven genes (*hxt1* through *hxt7*) are known to encode functional glucose transporters. These HXT proteins belong to the major facilitator family (MFS) of transporters and transport their substrate by passive, energy-independent facilitated diffusion (Marger *et al.,* 1993). *hxt2*, *hxt6* and *hxt7* encode high-

affinity transporters. *hxt5* is a moderate-affinity glucose transporter. *hxt1*, *hxt3*, and *hxt4* encode low-affinity glucose transporters (Reifenberger *et al.,* 1997). *gal2* encodes a galactose transporter. *snf3* and *rgt2* are the most divergent members of the glucose transporter gene family. They encode proteins with 12 predicted membrane-spanning domains and unusually long C-terminal tails (Özcan and Johnston, 1996). Several key experiments indicate they function as glucose sensors instead of glucose transporters. First, *snf3* and *rgt2* are required for *hxt* gene regulation by glucose (Özcan and Johnston, 1995). SNF3 is required for induction of *hxt2* and *hxt4* expression by low levels of glucose, indicating that it functions as a sensor of low levels of glucose. RGT2 appears to be a sensor of high levels of glucose because it is required for maximum induction of the low-affinity glucose transporter *hxt1*. Second, one mutation that alters a conserved amino acid (Arg to Lys) in SNF3 and RGT2 cause constitutive *hxt* gene expression (Özcan *et al.,* 1996). Third, the long C-terminal tails of SNF3 and RGT2 were demonstrated to be critical for glucose sensing (Özcan and Johnston, 1996).

Glucose regulation mainly occurs at the transcription level and it has two major effects on gene expression in *S. cerevisiae* (Gancedo, 1998). First, it represses transcription of many genes, including genes in the respiratory pathway (TCA cycle, the glyoxylate cycle and electron transport chain protein) and genes for utilization of other carbon sources (e.g. galactose, sucrose and maltose). This regulatory phenomenon is known as carbon catabolite repression (Gancedo, 1998). Second, glucose induces genes required for glucose utilization, such as genes encoding glycolytic enzymes and glucose transporters. The understanding of glucose sensing and signaling pathways in yeast has

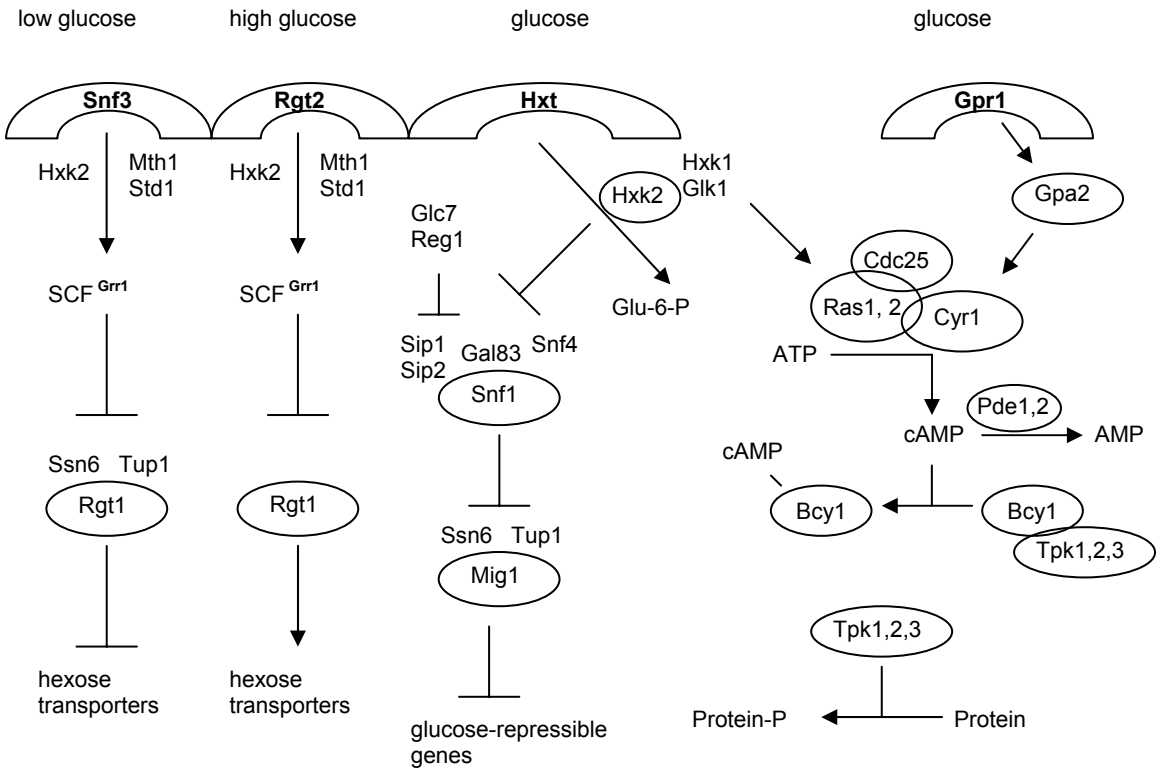


Figure 1.2 Mechanism of glucose repression and induction in *S. cerevisiae* (Rolland *et al.,* 2002).

progressed in recent years and four distinct signaling cascades have been proposed to be involved in the glucose regulatory machinery in the budding yeast *S. cerevisiae* (Rolland *et al.,* 2001; Rolland *et al.,* 2002) (Figure 1.2).

In *S. cerevisiae*, the hexokinase (HXK2) dependent carbon repression pathway is responsible for long term glucose repression (Figure 1.2), which contains several central components: MIG1, a DNA-binding zinc-finger protein that functions as transcription repressor; SNF1, a protein kinase complex; GLC7, protein phosphatase 1 and its regulatory subunit REG1. MIG1 recruits the general co-repressor proteins SSN6 and TUP1, binding to the promoters of glucose-repressible genes to inhibit their transcription (Treitel and Carlson, 1995). In *S. cerevisiae*, glucose suppresses the activity of SNF1 kinase; SNF1 activity increases 200-fold when glucose is removed (Wilson, 1996). Activated SNF1 kinase phosphorylates MIG1, which leaves the nucleus and derepresses glucose-repressed genes (Treitel and Carlson, 1995). The activating subunit SNF4 is required for SNF1 activity while the Sip1, Sip2 and Gal83 work together to maintain association of Snf4 with Snf1 kinase (Rolland *et al.,* 2001). The signal that affects SNF1 is unknown. One possible candidate is AMP (or AMP: ADP or ADP: ATP ratio) (Johnston 1999). The low-affinity glucose transporter *hxt1* and/or other transporters transport large amounts of glucose that are converted to glucose-6-phosphate by *hxk2* (hexokinase 2). ATP, produced through fermentation, inhibits the activity of SNF1 kinase (Johnston, 1999). The GLC7-REG1 protein phosphatase is also involved in regulating SNF1 function (Johnston, 1999).

The second pathway possesses two sugar sensors (SNF3 and RGT2) that mainly control the expression of functional hexose carriers. Three key components are identified in this pathway (Figure 1.2): RGT1, a transcription repressor and activator; SCF^{Grr1}, a multiprotein complex that modifies RGT1 activity; and two glucose sensors (SNF3 and RGT2) (Rolland *et al.,* 2001). Without glucose, RGT1 binds to *hxt* promoters and represses their expression. The general repressors SSN6 and TUP1 also participate in this process (Özcan and Johnston, 1996). At low levels of glucose, SNF3 protein is believed to bind to glucose and change its conformation, which causes SCF^{Grr1} complex to inactivate RGT1 repressor through the protein ubiquitin proteolysis pathway (Li and Johnston, 1997). At high concentrations of glucose, *rgt2* triggers transcription of a low affinity glucose transporter (Hxt1). This involves phosphorylation of Rgt1 to convert Rgt1 from a repressor to an activator (Mosley *et al.,* 2003). Grr1 is required for both low glucose induced inactivation and high glucose induced conversion of Rgt1 (Özcan *et al.,* 1996). The signal generated by SNF3 and RGT2 is unknown; however, two proteins (STD1 and MTH1) have been demonstrated to interact with the hydrophilic C-terminal tails of SNF3 and RGT2. Genetic analysis suggests that they are involved in transmission of the SNF3/RGT2 signal to stimulate hexose transporter gene expression (Schmidt *et al.,* 1999; Lafuente *et al.,* 2000).

In *S. cerevisiae*, the cAMP signaling cascade plays a central role in the control of metabolism, stress resistance and proliferation. The cAMP pathway is activated when glucose is added to cells growing on non-fermentable carbon sources or to stationary cells. It has been demonstrated that glucose is detected through a dual sensing process: a

hexokinase dependent intracellular detection following glucose uptake by Hxt genes and an extracellular glucose sensing by a G-protein coupled receptor (GPCR) system involving the Gpr1 and Gpa2 genes (Rolland *et al.,* 2002) (Figure 1.2). How these two processes activate adenylate cyclase (Cyr1) is still unknown; however, two G-proteins (Ras 1 and Ras 2) and their regulatory subunit (Cdc25) are essential for adenylate cyclase activity. The generated cAMP stimulates cAMP-dependent protein kinase A (PKA) by binding its regulatory subunit (Bcy1), thus releasing the catalytic protein kinase subunits (Tpk1, Tpk2 and Tpk3). The activated Tpk subunits phosphorylate downstream targets to activate glycolytic genes and inhibit genes involved in gluconeogenesis. In addition, PKA also triggers expression of several family-specific transcription factors enabling a coordinated regulation of gene families, which actually accounts for many of pleiotropic effects of the PKA pathway. The fourth pathway is not well defined and appears to utilize intermediate metabolites of glycolysis as metabolic messengers.

The *rco-3* mutant was originally isolated due to elevated expression of the conidiation-specific gene *con-10* (Madi *et al.,* 1994). Subsequent cloning and characterization of the *rco-3* gene revealed that it is involved in glucose repression, glucose transport and conidiation in *N. crassa* (Madi *et al.,* 1997). Several previously isolated *2-deoxyglucose resistant* (*dgr-1, dgr-2* and *dgr-3*) and/or *sorbose resistant (sor*) mutants (Perkin *et al.,* 1982; Allen *et al.,* 1989) are phenotypically similar or even identical to the *rco-3* mutant. Based on the phenotypic resemblance, it is possible these genes participate in the same genetic pathway as *rco-3*.

Here, I present further characterization of *rco-3* and *dgr-1* mutants in glucose transport, carbon regulation and conidiation. A suppressor isolation approach has identified three distinct genes that suppress the sorbose resistance phenotype of the *rco-3* mutant. All suppressors repair the regulation defect of the *rco-3* strain. To further elucidate the epistatic relationship among *rco-3*, *dgr-1* and suppressors, *rco-3*; *dgr-1*and *dgr-1* strains carrying suppressor mutation were generated and characterized.

Previous studies have demonstrated that *rco-3* is required for induction of glucose transport activities and the *rco-3* mutant displays different expression pattern of several glucose repressed genes (Madi *et al.,* 1997). Therefore, understanding glucose regulation of gene expression by transcriptional profiling in *N. crassa* will provide a basis to further elucidate the *rco-3* signaling cascades. A cDNA microarray containing 1363 unique genes (approximately 14% of *N. crassa* genes) was generated in collaboration with Dr. Deborah Bell-Pedersen's laboratory and the Laboratory for Functional Genomics in the Department of Biology. This microarray slide was used to examine the gene expression pattern of *N. crassa* grown in the presence of glucose or starved for glucose for two hours. 17% of the genes on the array were differentially regulated in response to glucose deprivation. Carbon metabolism, amino acid metabolism, lipid metabolism and genes involved in transport and the general translation machinery are coordinately regulated upon glucose depletion to adapt to the glucose limited environment. The microarray data indicate that *N. crassa* utilizes a significant fraction of glucose through fermentation in aerobic culture, a finding that is consistent with previous measurements of ethanol production by *N. crassa*.

The same microarray was used to examine gene expression patterns in *rco-3* and *dgr-1* strains under glucose repression and derepression conditions. The study indicates the *rco-3* and *dgr-1* mutants are defective in general carbon repression. *rco-3* and *dgr-1* are required for both induction and repression of glucose-regulated genes. The similar gene regulation profile and phenotypic resemblance of *rco-3* and *dgr-1* mutants demonstrated that they are very likely in the same signal transduction cascade, which is consistent with the genetic analysis of epistatic relationship between *rco-3* and *dgr-1* strains. In addition, *SP1A4*, representing a gene that is homologous to hexose transporters, was found to be differentially regulated by glucose, *rco-3* and *dgr-1* from microarray experiments. Disruption and overexpression of *SP1A4* demonstrates that it encodes a high affinity glucose transporter gene in *N. crassa*.

CHAPTER II

GLOBAL ANALYSIS OF THE RESPONSE OF *NEUROSPORA CRASSA* TO GLUCOSE STARVATION

OVERVIEW

Glucose is the preferred carbon source for most eukaryotic cells and glucose availability regulates many cellular behaviors. Glucose sensing, regulation of gene expression and metabolism have been extensively studied in *Saccharomyces cerevisiae* but are less well understood in filamentous fungi. Here, I present an analysis of the transcriptional profile of 1363 *Neurospora crassa* genes of wild type cell grown in the presence of glucose or starved for glucose for two hours. The major transcriptional response was observed for genes involved in central metabolism and growth. I found that 17% of the 1363 genes represented on the cDNA microarray were differentially regulated in response to glucose deprivation. The expression patterns of several genes were validated by Northern blot analysis and correlated with previous measurements of gene expression and enzyme activities. Comparing *N. crassa* profiling data with the published diauxic shift data from *S. cerevisiae* (DeRisi *et al.,* 1997) revealed that *S. cerevisiae* and *N. crassa* share a similar, but not identical, transcriptional response pattern for genes belonging to central carbon metabolism. The results indicate that *N. crassa* utilizes a significant fraction of glucose through fermentation to ethanol in aerobic culture, a finding that is consistent with previous measurements of ethanol production by *N. crassa*. The anaerobic fermentation of glucose to ethanol in an obligate

aerobe suggests that the strategy for glucose utilization in *N. crassa* differs from yeast and other previously characterized filamentous fungi and may relate to the ecological niche occupied by *N. crassa*. *N. crassa* may also serve as a useful model system for metabolic engineering of bioethanol production from plant substrates.

INTRODUCTION

The filamentous fungus *N. crassa* and the yeast *S. cerevisiae* serve as two model systems for the study of eukaryotic genetics, contributing to the fundamental understanding of cellular physiology, differentiation and development. These ascomycete fungi have been estimated to have diverged from each other more than 400 million years ago (Taylor *et al.,* 1999). The *N. crassa* genome consists of about 40 million base pairs (MB) (Galagan *et al.,* 2003) encoding approximately 10,000 proteins, while the *S. cerevisiae* genome is 12 MB. The larger genome size of *N. crassa* reflects the much greater morphological and developmental complexity of *N. crassa* (Springer 1993). In addition, *N. crassa* is typically thought to flourish as a pioneer species, rapidly colonizing newly heat-killed plant material, and so may use different strategies for carbon assimilation than *S. cerevisiae* and typical soil saprophytes. Despite these differences, the basic enzymatic steps involved in glucose metabolism are highly conserved.

Glucose is metabolized by a set of conserved enzymatic reactions. Respiration (aerobic) and fermentation (anaerobic) are two pathways for glucose utilization. Respiration proceeds at a low rate with a high yield of ATP, whereas fermentation

operates at a higher rate but with low ATP yield. In fermentation glucose is converted to ethanol rather than being completely degraded into carbon dioxide and water as it is via respiration. Complete oxidative phosphorylation requires the full participation of the TCA cycle to generate NADH from acetyl-CoA. The glyoxylate cycle is highly coordinated with the TCA cycle and can replenish intermediates lost from the TCA cycle. Together these pathways provide carbon for gluconeogenesis by supplying oxaloacetate for conversion to phosphoenoylpyruvate. In *S. cerevisiae,* the preferred mode of glucose metabolism is fermentative, resulting in the formation of ethanol, while most eukaryotic organisms primarily exploit glucose via respiration. Recent studies indicate that filamentous fungi, such as *Trichoderma reesei* (Chambergo *et al.,* 2002) and *Aspergillus nidulans* (Kelly *et al.,* 1990; Lockington *et al.,* 1997), exploit glucose primarily through respiration. *N. crassa* has been reported to generate ethanol in glucose medium in aerobic culture, and ethanol accumulation is proportional to the initial glucose concentration (Colvin *et al.,* 1973b). Therefore, the obligate aerobe, *N. crassa*, carries out respiration, but also utilizes glucose via anaerobic fermentation to ethanol at a much higher rate than the other studied filamentous fungi.

Glucose also serves as a signal to affect many properties of cellular physiology. The mechanisms by which *S. cerevisiae* senses the presence of glucose, regulates the expression of genes required for glucose uptake and metabolism, and represses transcripts for utilization of alternative carbon sources has been studied intensively (Reviewed by Gancedo, 1998; Rolland *et al.,* 2001; Rolland *et al.,* 2002). These mechanisms can be compared between *S. cerevisiae* and filamentous fungi. Distinct

glucose signaling cascades have been proposed for glucose regulatory effects in *S. cerevisiae* (Rolland *et al.,* 2001). Among these pathways, MIG1 complexes with SSN6 and TUP1 to mediate repression of a large number of glucose-regulated genes. RGT1 is a transcriptional regulator that acts downstream of two glucose sensors (SNF3 and RGT2) and mainly controls the expression of functional glucose carriers (Rolland *et al.,* 2002). Several apparent orthologs of the yeast glucose signaling pathways have been identified in *N. crassa* (Ebbole 1998). CRE1 is homologous to MIG1 and binds to the MIG1 consensus binding site (Serna *et al.,* 1999). The ortholog of *cre-1* in *A. nidulans*, *creA* has been demonstrated to function as the major repressor involved in glucose repression (Mathieu and Felenbok, 1994; Klein *et al.,* 1998). *rco-1* (*regulation of conidiation*) is orthologous to *Tup1*, and encodes a cell type-specific repressor (Yamashiro *et al.,* 1996). However, no role for *rco-1* in glucose regulation has been discovered. This was also found to be the case with the *rco-1* homolog of *A. nidulans*, *rcoA*, that has a profound affect on growth and development but little, if any, effect on CREA-mediated carbon catabolite repression (Hicks *et al.,* 2001). RCO-3 is a counterpart of SNF3 and RGT2 (Madi *et al.,* 1997). The *rco-3* gene appears to encode a sugar sensor and is required for the full induction of glucose transporter activity (Madi *et al.,* 1997). Thus, although homologs of some yeast genes do not appear to play important roles in glucose regulation in filamentous fungi, others do.

The completion of *N. crassa* genome sequence permits the investigations based on global analysis of gene expression patterns to study aspects of glucose catabolism at a greater resolution than previously possible. To gain a more comprehensive

understanding of glucose metabolism and regulation in *N. crassa*, a cDNA microarray chip with 1363 unique transcripts representing approximately 14% of *N. crassa* genome was generated. The change in the gene expression profile that accompanies the adaptation of *N. crassa* from glucose repression (2% glucose) to glucose derepression (medium lacking glucose) was examined. Analysis of this shift from glucose sufficiency to glucose limitation is expected to reveal the transcriptional response of genes for central metabolism in response to glucose levels and the response of genes involved in growth rate control as cells are deprived of carbon source.

N. crassa has been characterized for several decades as a model system for biochemical genetics. Thus it was possible for us to compare microarray results to previous measurements of mRNA and enzyme activity levels. I also compared the patterns of transcript abundance in *N. crassa* with the previously reported data for the diauxic shift for *S. cerevisiae* (DeRisi *et al.,* 1997) and data for *T. reesei* (Chambergo *et al.,* 2002). These comparisons allowed me to assess the strategy for glucose utilization in *N. crassa* relative to other filamentous fungi and *S. cerevisiae*. My microarray results are consistent with previous work showing that *N. crassa* is unique in its ability to carry out simultaneously high rates of respiration and anaerobic fermentation.

MATERIALS AND METHODS

Strains and growth conditions

Strain Oak Ridge 74OR23-1A (FGSC no. 987), obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas, Kansas City, Kansas, was used for

microarray and Northern blot hybridizations. Unless indicated, strains were grown in Vogel's minimal medium (refer as minimal medium throughout) (Davis and De Serres, 1970) supplemented with 2% glucose as described previously (Madi *et al.,* 1997). For glucose repression and derepression experiments, conidia were collected with sterile water through Miracloth (Calbiochem) by filtration from a 50 ml solid medium culture after six days of growth. Conidia were counted using a hemocytometer (Hausser Scientific) and inoculated at 10^6 conidia / ml into 50 ml liquid minimum medium containing 2% peptone and 2% glucose. Liquid cultures were grown approximately 17-18 hours on a rotary shaker (200 rpm) at 34°C. The mycelium was harvested by vacuum filtration on sterile Whatman filter paper (VWR International) and cut into two pieces of approximately equal size with a sterile razor blade. The halves of each mycelia pad were inoculated in parallel into 50 ml fresh 2% glucose-supplemented minimal medium (glucose repression) or the same medium without glucose (glucose derepression). Since peptone can suppress the derepression of conidiation in submerged culture (Madi *et al.,* 1997), I also inoculated the mycelia pads into minimal medium plus 2% peptone with or without 2% glucose. The inoculated flasks were vigorously shaken to disperse the cells and incubated for two hours at 34°C. Cultures were harvested by filtration and frozen in liquid nitrogen prior to RNA extraction. In order to reduce the variation arising from an individual growth experiment, mycelium was harvested from five independent cultures using the same growth conditions and combined for mRNA extraction for microarray experiments.

Nucleic acid isolation, hybridization and sequencing

Genomic DNA isolation, RNA extraction and Northern blot hybridization were performed as described previously (Madi *et al.,* 1994). Northern blot analyses use RNA preparation from one growth culture, whereas microarray experiments use the combined RNA preparation from multiple cultures. mRNA was isolated from total RNA using polyATtract mRNA isolation system IV kit (Promega). DNA probes for NCU10021.1 (SP1A4), NCU06358.1 (NP4E6), NCU09873 (phosphoenoylpyruvate carboxykinase), *eas*, and *grg-1* were obtained from the corresponding sequence-verified EST clones by PCR. Actin probe was amplified from genomic DNA using specific primers. RNA quality and loading were verified by hybridization with an rDNA probe. Sequencing of cDNA clones was performed using the Prism dideoxy sequencing kit (Applied Biosystem) with T3 and T7 primers.

Microarray construction and hybridization

EST (expressed sequence tag) libraries representing different stages of the *N. crassa* life cycle (germinated conidia, mycelia and sexual stage) were provided by the *Neurospora* genome project at the University of New Mexico (Nelson *et al.,* 1997). 1764 EST sequences from three EST libraries were assembled using STACKPACK^{TM} software (Electric Genetics). BLAST analysis of the EST sequences against the newly released *N. crassa* genomic sequence from the WhiteHead Institute indicated that 1363 unique genes were represented, accounting for approximately 14% of all *N. crassa* genes. The difference between the number of ESTs and the unigene set is due to multiple representations of genes in our EST collection. cDNA inserts were amplified by PCR in

96-well format using universal T3 and T7 primers by our laboratory and Dr. Deborah Bell-Pedersen's laboratory. PCR products were verified by agarose gel electrophoresis. Purified PCR products were dissolved in 3 X SSC and printed on poly-lysine slides in the Laboratory for Functional Genomics in the Department of Biology (Lewis *et al.,* 2002). Genes from other organisms - *Arabidopsis thaliana pap-1* and the *verK* gene from *Aspergillus nidulans*, were placed on the microarray slide as negative controls (Lewis *et al.,* 2002).

The two channel (Cy3 and Cy5) labeling system, which allows simultaneous comparison of gene expression patterns between two samples (DeRisi *et al.,* 1997), was used. mRNA (300 ng) from each sample was reverse transcribed and hybridized to the microarray following the two-step protocol from 3DNA^{TM} Submicro EX Expression Array Detection Kit (Genisphere). The TIFF images were obtained using an Affymatrix 426^{TM} Array Scanner (Affymatrix). In considering photobleaching of the fluorescent dyes, especially Cy5, the scanner laser power and photo-multiple tube (PMT) settings were adjusted to balance the signals in the two channels. The TIFF images of each channel were imported into ScanAlyze to obtain raw signal intensities for each spot (ScanAlyze was written by Michael Eisen).

Expression data analysis

Four replicate microarray experiments of the wild type strain under glucose repression and derepression condition were obtained. In order to assess dye bias effects, two sets of data were obtained in which cDNA from cells grown in glucose-rich medium were labeled with Cy3, whereas cDNA from cells starved for glucose for two hours were

labeled with Cy5. Two additional dye-swap experiments were performed in which the fluorescent dyes were switched during the labeling process. The tab-delimited data files from ScanAlyze were analyzed using GeneSpring (Silicon Genetics) for advanced analysis. First, the background value was subtracted from the raw data of each spot. Then, the subtracted expression data was filtered based on the value derived from the average value of the negative control spots to eliminate any spots that display signal intensity below the average value of negative control spots. The resulting data value was normalized using the intensity dependent (Lowess) normalization protocol, in which 20.0% of the data was used to calculate the Lowess fit at each point and the control value for each measurement was adjusted on the curve (GeneSpring Software, Silicon Genetics).

Duplicate experiments were analyzed as individual experiments and reproducibility of replicate arrays was assessed using an experiment tree analysis in which the correlation coefficient of each gene across the set of experiments was quantitated to build the tree. The dye swap replicate experiments always branched together in the experiment tree indicating there is a subtle dye bias effect. The overall correlation coefficient was 0.85. Genes with two-fold or higher level changes in at least three of four duplicate experiments were flagged; this subset of genes was further filtered to identify genes that had a minimum two-fold differentiation in their averaged normalized value.

RESULTS

Global transcription response of N. crassa during glucose deprivation

A cDNA microarray for *N. crassa* containing 1764 clones from the New Mexico EST libraries (Nelson *et al.,* 1997) was produced (Lewis *et al.,* 2002). Comparison of the cDNA sequences with the recently released *N. crassa* genome sequence from the Whitehead Institute (Galagan *et al.,* 2003) confirmed that 1363 unique genes are present in the microarray, with 401 genes being represented by more than one EST. These multiple copies provide one level of control to measure the reliability of the microarray results. Among these genes, 858 have corresponding homologs in *S. cerevisiae* (BLAST e values < e^{-5}).

Four replicate microarray hybridizations were performed (see Materials and Methods). The correlation coefficient between these experiments was greater than 0.85, indicating a high degree of reproducibility between the experiments. Hybridization signals that were at least 2-fold over background were used to calculate the ratio of the signal in glucose starved to glucose grown cultures. The data are represented as the average ratio of glucose deprived to glucose grown cells for all experiments (two samples of each cDNA per array with four array hybridizations). The transcript levels of 118 different genes (8.7% of the total) increased under glucose limitation by a factor of 2 or more. A nearly fraction (7%) of transcript levels decreased upon glucose deprivation. Those ESTs with sequence similarity to protein sequences in the NCBI non-redundant data base were assigned to functional groups according to MIPS functional categories (Figure 2.1). Not surprisingly, the largest groups of known transcripts in both lists

represent genes involved in metabolism. Genes whose transcript levels decreased with glucose starvation belong to general biosynthetic processes including amino acid biosynthesis (11%) and protein synthesis (7%), reflecting the reduction in growth rate that accompanies the adaptation to growth in the absence of glucose. Consistent with this finding, I observed a decline in transcripts of genes encoding components of the cytoskeleton (6%). In contrast, the expression of genes involved in the degradation of proteins, nucleotides, lipids, and amino acids (5%) are elevated upon glucose depletion. Thus, processes for biomass production (biosynthesis and cytoskeleton) are down-regulated in the adaptation to glucose limitation and expressions of genes for utilization of alternative carbon sources are up-regulated to allow resumption of growth using these new nutrient sources.

Gene expression patterns in N. crassa: Central metabolism

Glycolysis, the tricarboxylate acid (TCA) cycle, the glyoxylate cycle, and gluconeogenesis are essential for glucose utilization and this central metabolism is conserved in eukaryotic cells. Glucose is converted to pyruvate via glycolysis after it is absorbed. Pyruvate occupies a branch point for fermentation, gluconeogenesis and the TCA cycle (and ultimately respiration), which are mediated respectively by pyruvate decarboxylase, phosphoenolpyruvate carboxykinase and pyruvate dehydrogenase. The mRNA levels for genes encoding most glycolytic enzymes decline rapidly after the downshift from glucose-rich to glucose-deficient medium. Conversely, transcripts for many of the genes involved in the TCA and glyoxylate cycles increase in response to the

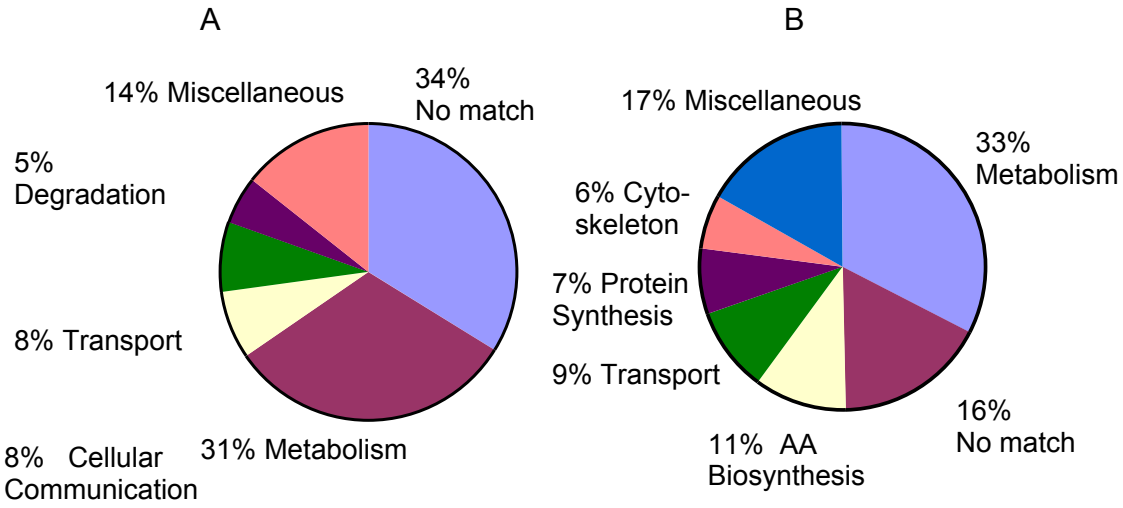


Figure 2.1 Summary of the distribution of 213 *N. crassa* genes differentially regulated by glucose. (A) 118 transcripts were induced under glucose deprivation in wild type strain. (B) 95 genes were repressed under glucose deprivation in wild type strain. The classification follows MIPS functional categories with BLASTP hits (P<1e-5) to *S. cerevisiae* and the NCBI non-redundant database. No match represents sequences with no significant similarity to proteins in the database. The Miscellaneous category refers to genes involved in a variety of different processes and includes genes with unknown function.

shift to glucose-deficient medium (Table 2.1). This shift in central metabolism is similar to the response of *S. cerevisiae* to glucose deprivation (DeRisi *et al.,* 1997) (Figure 2.2).

Twenty four ESTs representing 12 genes involved in 9 of the 11 steps in glycolysis are present in our microarray. These genes display either down-regulation or are unaffected by the down-shift to glucose deficiency (Table 2.1). A direct comparison of the regulation pattern of the *N. crassa* and *S. cerevisiae* orthologs shows a similar pattern. This is expected since in the absence of glucose there is virtually no substrate available for glycolysis and a reduced need to produce additional enzymes. In particular, transcript level for a gene encoding an enzyme that catalyzes an irreversible step, pyruvate kinase, was reduced six-fold. One notable difference between *S. cerevisiae* and *N. crassa* is that in *N. crassa,* the transcription of the genes encoding the pyruvate dehydrogenase complex is 5-fold higher in the presence of glucose than in its absence. This finding suggests that in *N. crassa* the flow of pyruvate into the TCA cycle, and ultimately respiration, is being promoted at the same time that flow into fermentation is highly active.

It has been well documented that *N. crassa* produces ethanol through fermentation of glucose in aerobic culture conditions (Perlman, 1950; Colvin *et al.,* 1973b). Consistent with this observation, I found that transcript levels for pyruvate decarboxylase, which catalyzes the unidirectional rate-limited step of fermentation, was five-fold higher in glucose-rich medium, as previously reported (Alvarez *et al.,* 1993). The acetaldehyde generated by pyruvate decarboxylase may be efficiently reduced to ethanol by NADH through a constitutively expressed alcohol dehydrogenase of *N.*

crassa with most sequence similarity to *S. cerevisiae ADH1* (Table 2.1). Alcohol dehydrogenase II and aldehyde dehydrogenase were expressed at a relatively low level (Table 2.1) in the presence of glucose, suggesting that alcohol could accumulate in *N. crassa* cultures. This regulatory pattern is similar to that in *S. cerevisiae*.

As mentioned above, the elevated transcript levels of the pyruvate dehydrogenase gene in glucose-rich medium suggests that flux of pyruvate into the TCA cycle is robust even when fermentation is active. Our microarray contains 16 genes encoding enzymes involved in catalyzing all eight steps in the TCA cycle and five of the six steps in the glyoxylate cycle. In contrast to the increased transcript levels for all of the steps of the TCA cycle in *S. cerevisiae*, several of the genes encoding enzymes of the TCA cycle in *N. crassa* are not highly regulated by a glucose down-shift. Genes for isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, succinyl-CoA ligase and fumarase are unaffected or even down-regulated (Figure 2.2) (Table 2.1). The genes encoding the remaining enzymes of the TCA cycle are up-regulated after transfer to glucose-free medium (Figure 2.2) (Table 2.1). In *N. crassa*, as in *S. cerevisiae*, a large increase in transcript levels of genes of the glyoxylate cycles is observed upon glucose exhaustion. This is consistent with previous reports that enzyme activities in the glyoxylate cycle were elevated in a downshift to acetate medium (Flavell and Fincham, 1968; Flavell and Woodward, 1970a; Flavell and Woodward, 1970b; Flavell and Woodward, 1971a). The expression patterns of genes encoding enzymes in central metabolism suggest *N. crassa* exploits glucose simultaneously through fermentation and respiration in aerobic

Table 2.1 *N. crassa* genes participate in central metabolism.

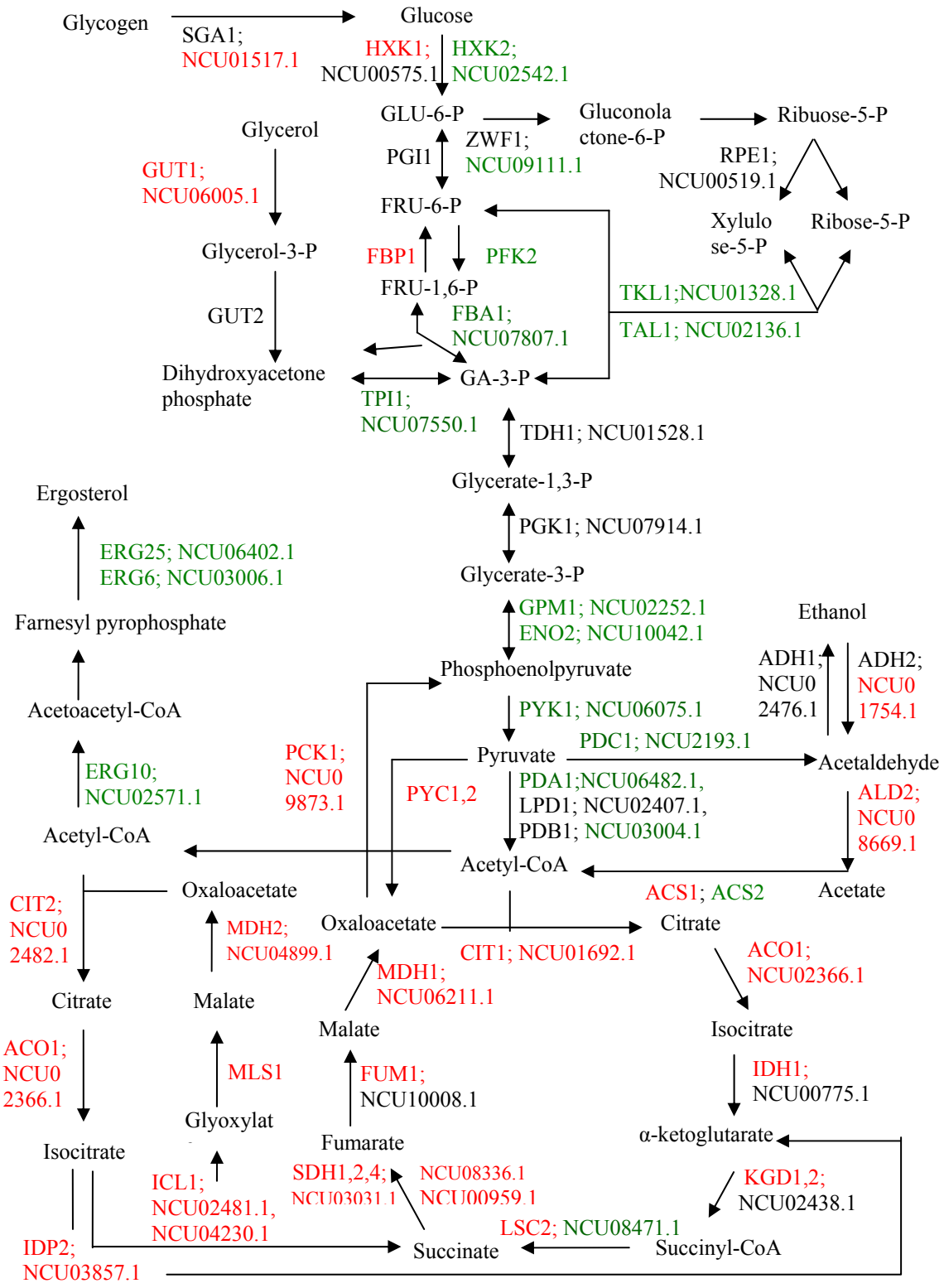
Ncr ORF [a]	EST ID [b]	Blast Match [c]	Ncr Fold [d]	Sce Homolog [e]	Sce Fold [f]
Glycolysis/Gluconeogenesis					
NCU06482.1	W17H2	Pyruvate dehydrogenase	0.21	Pda1	1.5
NCU03004.1	SC5E2	Pyruvate dehydrogenase	0.21	Pdb1	0.9
NCU02407.1	NM6A1	Dihydrolipoamide dehydrogenase	0.6	Lpd1	1.7
NCU06075.1	SM2G11	Pyruvate kinase	0.17	Pyk1	0.2
NCU06075.1	SC2A6	Pyruvate kinase	0.16	Pyk1	0.2
NCU06075.1	NC4C8	Pyruvate kinase	0.18	Pyk1	0.2
NCU10042.1	SC1H2	Enolase	0.35	Eno2	0.42
NCU10042.1	SP6F12	Enolase	0.4	Eno2	0.42
NCU02252.1	NC2D12	Phosphoglycero mutase	0.18	NH [g]	
NCU02252.1	NC3B11	Phosphoglycero mutase	0.18	NH	
NCU02252.1	SC2H9	Phosphoglycero mutase	0.16	NH	
NCU07914.1	NC2D1	Phosphoglycerate kinase	1.4	Pgk1	0.7
NCU07914.1	NC3D6	Phosphoglycerate kinase	1	Pgk1	0.7
NCU01528.1	CCG-7	Glyceraldehyde 3-phosphate dehydrogenae	1.3	Tdh1	0.65
NCU01528.1	NC1G4	Glyceraldehyde 3-phosphate dehydrogenae	1.3	Tdh1	0.65
NCU01528.1	NC3C3	Glyceraldehyde 3-phosphate dehydrogenae	1	Tdh1	0.65
NCU01528.1	W08B11	Glyceraldehyde 3-phosphate dehydrogenae	1.2	Tdh1	0.65
NCU07550.1	SC1G8	Triosephosphate isomerase	0.43	Tpi1	0.46
NCU07550.1	NC2E4	Triosephosphate isomerase	0.44	Tpi1	0.46
NCU07807.1	SC3F5	Fructose-bisphosphate aldolase	0.19	Fba1	0.42
NCU02542.1	NC4B10	Hexokinase	0.49	Hxk2	0.45
NCU00575.1	W10H9	Glucokinase	0.54	Glk1	3.8
NCU09873.1	NM5C3	Phosphoenolpyruvate carboxykinase	33	Pck1	14
NCU09873.1	SM3H9	Phosphoenolpyruvate carboxykinase	29	Pck1	14
TCA Cycle					
NCU01692.1	NM6F5	Citrate synthase	4.5	Cit1	9.1
NCU02366.1	SC3F7	Aconitate hydratase	3.8	Aco1	6.3
NCU00775.1	SC1C8	Isocitrate dehydrogenase	1.2	Idh1	3.2
NCU02438.1	NM1B4	Alpha-ketoglutarate dehydrogenase	0.8	Kgd2	2.6
NCU02407.1	NM6A1	Dihydrolipoamide dehydrogenase	0.6	Lpd1	1.7
NCU08471.1	W13H10	Succinyl-CoA ligase	0.3	Lsc 2	4.2
NCU08336.1	NC1F7	Succinate dehydrogenase	3.5	Sdh1	4.2
NCU00959.1	NM7H6	Succinate dehydrogenase	5.3	Sdh2	6.3
NCU03031.1	SC5F1	Succinate dehydrogenase	4.2	Sdh4	3.3
NCU10008.1	NM1B3	Fumarase	1.3	Fum1	3.7
NCU04899.1	W08E4	Malate dehydrogenase	4.5	Mdh1	5.9
NCU04899.1	W08B12	Malate dehydrogenase	3.1	Mdh1	5.9

Table 2.1 (continued)

Ncr ORF [a]	EST ID [b]	Blast Match [c]	Ncr Fold [d]	Sce Homolog [e]	Sce Fold [f]
Glyoxylate Cycle					
NCU02482.1	NP6C12	Citrate synthase	6.8	Cit2	4.8
NCU02366.1	SC3F7	Aconitate hydratase	3.8	Aco1	6.3
NCU02481.1	NM3F4	Isocitrate lyase	2.9	Icl1	13
NCU04230.1	NP3E9	Isocitrate lyase	11	Icl1	13
NCU03857.1	W17F2	NADP-dependent isocitrate dehydrogenase	2.8	Idp2	9.6
NCU06211.1	NP2E7	Malate dehydrogenase	6	Mdh2	2.6
Fermentation					
NCU02476.1	NP6D7	Alcohol dehydrogenase	1.1	Adh1	0.5
NCU02193.1	NC3D2	Pyruvate decarboxylase	0.19	Pdc6	0.47
Pentose Phosphate Pathway					
NCU09111.1	NC3F8	Glucose-6-phosphate 1-dehydrogenase	0.38	Zwf1	1.3
NCU09111.1	W07G12	Glucose-6-phosphate 1-dehydrogenase	0.41	Zwf1	1.3
NCU09111.1	W09C4	Glucose-6-P-1-dehydrogenase	0.35	Zwf1	1.3
NCU00519.1	W13F7	D-ribulose-5-Phosphate 3-epimerase	1.4	Rpe1	1.4
NCU01328.1	NC4B12	Transketolase	0.33	Tkl1	0.32
NCU02136.1	NC3G5	Transaldolase	0.27	Tal1	0.32
NCU02136.1	W10A6	Transaldolase	0.46	Tal1	0.32
Mitochondria					
NCU00502.1	SC5G11	ATP synthase subunit 4	0.41	Atp4	2.6
NCU01689.1	W08D11	Mitochondrial carrier	0.38	Yhm2	0.35
NCU03561.1	SC5E1	Mitochondrial carrier	0.36	Yhm1	0.56
NCU03359.1	W08C12	Mitochondrial protein	3.34	Yme1	0.9
NCU02668.1	NM2F12	Mitochondrial protein	0.43	Sun4	0.35
NCU02668.1	W06F9	Mitochondrial protein	0.48	Sun4	0.35
NCU05259.1	SM4H12	Delta-9-fatty acid desaturase	0.19	Ole1	1
NCU05259.1	NC3E10	Delta-9-fatty acid desaturase	0.19	Ole1	1
NCU04173.1	W17E2	Actin	0.34	Act1	0.97

[a] The *N. crassa* predicted ORFs are from the Whitehead Institute *Neurospora* sequence project
[b] The *N. crassa* ESTs are from *Neurospora* genome project at the University of New Mexico
[c] The Blast Match represents the most significant match in the NCBI database (minimum e-value $1e^{-5}$)
[d] The *Ncr* fold is the average ratio of glucose deprived to glucose grown cells over four replicate experiments (eight spots).
[e] The *Sce* Homolog is the best hit using predicted *N. crassa* ORF to blast against the NCBI *S. cerevisiae* database.
[f] The *Sce* fold is from diauxic shift data previously reported by DeRisi *et al.,* 1997 (http://cmgm.stanford.edu/pbrown/explore/ diauxsearch.html)
[g] NH indicates the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

Figure 2.2 Comparison of central metabolism pathways in *S. cerevisiae* and *N. crassa*. Only the key metabolic intermediates are indicated. Double-headed arrows denote reversible reactions. The yeast genes encoding the enzyme that catalyze each step are identified by name and the corresponding *N. crassa* genes are indicated by the predicted ORF designation. Red letters indicate genes whose expression is induced in the diauxic shift (*S. cerevisiae*) or glucose starvation (*N. crassa*). Green letters indicate genes that are repressed in the diauxic shift (*S. cerevisiae*) or during glucose starvation (*N. crassa*). Black letters indicate genes unaffected by glucose in *S. cerevisiae* and *N. crassa.*


Glycogen
SGA1; NCU01517.1
Glucose
HXK1; NCU00575.1
HXK2; NCU02542.1
GLU-6-P
Gluconolactone-6-P
Ribuose-5-P
Glycerol
GUT1; NCU06005.1
PGI1
ZWF1; NCU09111.1
RPE1; NCU00519.1
FRU-6-P
Xylulose-5-P
Ribose-5-P
Glycerol-3-P
FBP1
PFK2
FRU-1,6-P
TKL1;NCU01328.1
TAL1; NCU02136.1
GUT2
FBA1; NCU07807.1
Dihydroxyacetone phosphate
GA-3-P
TPI1; NCU07550.1
TDH1; NCU01528.1
Glycerate-1,3-P
Ergosterol
PGK1; NCU07914.1
ERG25; NCU06402.1
ERG6; NCU03006.1
Glycerate-3-P
GPM1; NCU02252.1
ENO2; NCU10042.1
Ethanol
Farnesyl pyrophosphate
Phosphoenolpyruvate
ADH1; NCU02476.1
ADH2; NCU01754.1
Acetoacetyl-CoA
PYK1; NCU06075.1
PDC1; NCU2193.1
PCK1; NCU09873.1
Pyruvate
Acetaldehyde
ERG10; NCU02571.1
PYC1,2
PDA1;NCU06482.1, LPD1; NCU02407.1, PDB1; NCU03004.1
ALD2; NCU08669.1
Acetyl-CoA
Acetyl-CoA
Acetate
CIT2; NCU02482.1
Oxaloacetate
ACS1; ACS2
MDH2; NCU04899.1
Oxaloacetate
Citrate
CIT1; NCU01692.1
ACO1; NCU02366.1
Citrate
Malate
MDH1; NCU06211.1
Malate
Isocitrate
ACO1; NCU02366.1
MLS1
FUM1; NCU10008.1
IDH1; NCU00775.1
Glyoxylat
Fumarate
Isocitrate
α-ketoglutarate
ICL1; NCU02481.1, NCU04230.1
SDH1,2,4; NCU03031.1
NCU08336.1 NCU00959.1
KGD1,2; NCU02438.1
LSC2; NCU08471.1
IDP2; NCU03857.1
Succinate
Succinyl-CoA

conditions, which differs from the primarily fermentative utilization of glucose by *S. cerevisiae*.

Utilization of non-fermentable carbon source requires gluconeogenesis to generate glucose 6-phosphate, which can be channeled into the pentose phosphate pathway (PPP) to produce NADPH and ribose for biosynthesis of nucleic acids and nucleotide co-factors. The transcript level of phosphoenolpyruvate carboxykinase, the key gene in gluconeogenesis, was elevated thirty-fold upon glucose exhaustion in my experiments and this induction was verified by Northern Blot analysis (Figure 2.3). Upon exhaustion of glucose, the reduction of transcript levels of genes encoding glucose-6-phosphate 1-dehydrogenase, transketolase and transaldolase suggest a down-regulation of the PPP. The PPP provides important intermediate metabolites and cells can utilize glucose exclusively via the PPP in the event that glycolysis is blocked (Jacoby *et al.,* 1993). This reduction in transcripts involved in glycolysis, the PPP, and some of the TCA cycle likely reflects the reduced growth rate of cells during the adaptation to glucose deprivation. However, gluconeogenesis is highly induced to provide the carbon needed for growth on alternative carbon sources. Gluconeogenesis requires the activities of the glyoxylate pathway and the TCA cycle to produce oxaloacetate (Figure 2.2). I found that transcript levels of TCA cycle genes involved in gluconeogenesis were coordinately regulated with the genes for the glyoxylate pathway, but not with the other genes of the TCA cycle. This suggests that although the expressions of some of the genes of the TCA cycle are not regulated by glucose, several

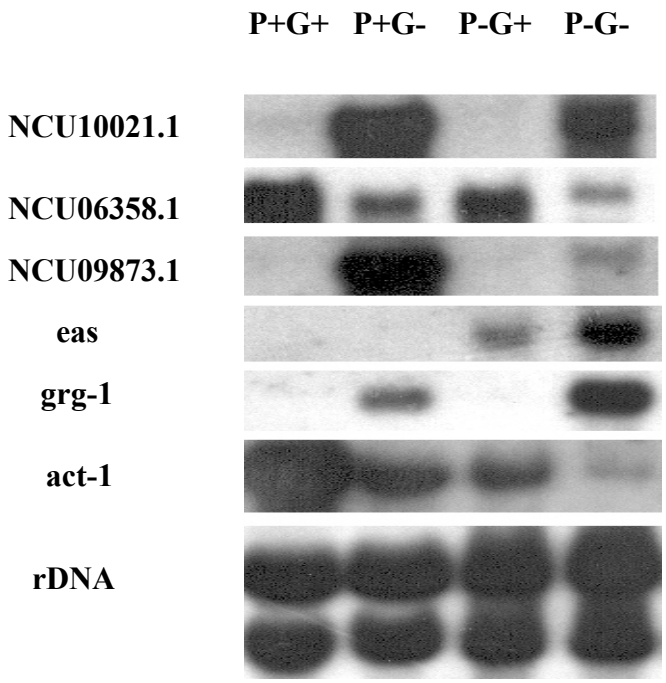


Figure 2.3 Northern blot analysis of glucose responsive genes. Several genes are differentially regulated by glucose and peptone in the wild type strain. Cultures were grown in minimal medium supplemented with 2% peptone and 2% glucose for approximately 18 hrs before transfer into four different media for an additional two hours. P+G+, minimal medium containing 2% glucose and 2% peptone, P+G-, minimal medium with 2% peptone but no glucose. P-G+, minimal medium with 2% glucose but no peptone. P-G-, minimal medium no carbon source. Genes that are not identified in *N. crassa* are indicated by predicted ORF number assigned by the Whitehead Institute (Galagan *et al.,* 2003). Previously identified *N. crassa* genes are represented by gene name.

enzymes of the TCA cycle are up-regulated along with the glyoxylate cycle to provide carbon for gluconeogenesis.

Lipid metabolism

N. crassa contains all typical major lipids found in eukaryotic membranes, such as phospholipids, sphingolipids, triacylglycerols and sterols. In *S. cerevisiae*, fatty acid degradation proceeds via beta-oxidation in the peroxisome whereas in *N. crassa* fatty acid degradation and the glyoxylate cycle enzymes are found co-localized in the glyoxysome (Fossa *et al.,* 1995).

The fatty acid degradation system was coordinately regulated under glucose depletion in *N. crassa*. First, fatty acids need to be activated and imported before they can be degraded, and this is carried out by FAA1, FAA2 and FAA4 in yeast (Fargeman, *et al.,* 2001). Although *faa1* and *faa4* homologs in *N. crassa* were only slightly induced, the *faa1* homolog was induced to a similar extent as in *S. cerevisiae* by glucose starvation, suggesting an increased ability to activate and import fatty acids when glucose was exhausted (Table 2.2). Second, the elevated expression of genes encoding MFP, dimeric 3-oxoacyl-CoA thiolases and SPS19 homolog indicate increased activities to degrade saturated and unsaturated fatty acids in the absence of glucose in *N. crassa* (Table 2.2). In addition, several other transcripts with sequence similarity to yeast genes involved in fatty acid degradation, including peroxisomal targeting signal receptor, peroxisomal biogenesis protein and ATP transporters, were coordinately regulated. For example, ant1 encodes an ATP transporter that transports cytoplasmic ATP into the

Table 2.2 *N. crassa* genes participate in lipid metabolism.

Ncr ORF [a]	EST ID	Blast Match	Ncr Fold	Sce Homolog	Sce Fold
Fatty Acid Degradation					
NCU08828.1	NP2E8	Peroxisomal hydratase-dehydrogenase	5.5	Fox2	1.6
NCU08828.1	NM4H8	Peroxisomal hydratase-dehydrogenase	5.1	Fox2	1.6
NCU04796.1	NM5E11	Peroxisomal 3-ketoacyl-CoA thiolase	4.5	Fox3	1.4
NCU03893.1	SP4C8	Peroxisomal 2,4-dienoyl-CoA reductase	2.4	Sps19	2.1
NCU04380.1	SP1D3	Long-chain fatty acid--CoA synthetase	1.2	Faa1	2.5
NCU01654.1	NM7A4	Acyl-CoA synthetase	2.7	Faa2	2.5
NCU03929.1	SP6C11	Long-chain fatty acid--CoA synthetase	1	Faa4	0.36
NCU00580.1	NM4B3	Acyl-CoA thioesterase	5.1	Tes1	2.2
NCU02960.1	NP5F8	Peroximal targeting signal receptor	3	Pas10	1.18
NCU07915.1	SM1D5	Peroxisomal organization and biogenesis protein	6.7	Yor292c	0.81
NCU00316.1	NM7A7	Adenine nucleotide transporter	4.7	Ant1	0.72
NCU03151.1	SC5D10	Peroxisomal membrane protein	0.3	Ahp1	0.5
Fatty Acid Synthesis					
NCU03492.1	NP6F6	fatty acid hydroxylase	0.42	Scs7	1.8
NCU05259.1	SM4H12	Delta-9-fatty acid desaturase	0.19	Ole1	1
NCU05259.1	NC3E10	Delta-9-fatty acid desaturase	0.19	Ole1	1
NCU07859.1	SM2D12	Cyclopropane-fatty-acyl-phospholipid synthease	0.45	NH [b]	
Ergosterol Biosynthesis					
NCU03006.1	NM5B12	Sterol c-methyltransferase	0.25	Erg6	0.3
NCU02571.1	NC4D1	Acetyl-CoA C-acetyltransferase	0.26	Erg10	0.48
NCU02571.1	SC1A4	Acetyl-CoA C-acetyltransferase	0.18	Erg10	0.48
NCU06402.1	SC2F1	C-4 sterol methyl oxidase	0.3	Erg25	0.33
Lipid Transport					
NCU02263.1	W17F1	Phosphatidylinositol transfer protein	0.49	Sec14	1.9
NCU03372.1	SP3B10	Sterol carrier protein	6.46	NH	

[a] The columns in this table are the same as Table 2.1.
[b] NH indicates the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

peroxisomal lumen in exchange for AMP generated during activation of fatty acids (Palmieri *et al*., 2001) and transcript level of the *ant1* homolog in *N. crassa* was elevated upon glucose starvation (Table 2.2). Overall, transcript levels for fatty acid degradation genes appeared to be more highly elevated in *N. crassa* compared to *S. cerevisiae* in response to glucose limitation, suggesting that transcriptional control is an important mechanism for coordinating expression of genes for beta-oxidation in *N. crassa*.

In contrast to the induction of fatty acid degradation genes, transcript levels of genes encoding enzymes involved in lipid biosynthesis were significantly reduced upon glucose deprivation. *ole1* encodes delta-9-fatty acid desaturase, a key enzyme for unsaturated fatty acid biosynthesis in *S. cerevisiae*. An *OLE1* homolog in *N. crassa*, was strongly repressed by glucose starvation. Another example is the sterol biosynthesis pathway. Sterols are essential lipid components of eukaryotic membranes and the primary fungal sterol is ergosterol. The low expression of genes encoding enzymes homologous to yeast ergosterol biosynthetic genes (*erg10, erg6, erg25*) is consistent with the inhibition of sterol biosynthesis upon glucose depletion in *N. crassa* (Figure 2.2) (Table 2.2).

Amino acid metabolism

The reduction of growth rate in response to the glucose downshift and the need to utilize amino acids to provide an alternate carbon and energy source leads to the expectation of a transcriptional shutdown of amino acid biosynthetic genes and an increase in expression of genes for amino acid catabolism. It has been shown that amino acid biosynthetic gene pathways are under control of a global activator. This regulatory

phenomenon is termed cross-pathway control and is mediated through CPC1 in *N. crassa* (Paluh *et al.,* 1988) and GCN4 in *S. cerevisiae* (Hinnebusch *et al.,* 1988). The down- regulation of *cpc-1* upon glucose limitation may be responsible, at least in part, for the observed down-regulation of many of the genes for amino acid biosynthesis (Table 2.3). Interestingly, *gcn4* was not down-regulated in *S. cerevisiae*, despite the fact that transcript levels for amino acid biosynthetic pathways were reduced.

Several amino acid biosynthetic pathways represented by multiple genes on the microarray were coordinately down-regulated under glucose starvation (Table 2.3). One example is the sulfur assimilation and methionine biosynthesis pathways, which have been well-characterized in *N. crassa* (Marzluf, 1997). Sulfate is transported into the cell by sulfur permease and converted to methionine and cysteine. Control of the structural genes that specify the enzymes for sulfur metabolism occurs mainly at the transcription level by the sulfur regulatory circuit in *N. crassa* (Marzluf, 1997). My studies indicate that the sulfur assimilation circuit was coordinately down-regulated when cells were subject to glucose-limiting condition. Seven of the eight genes on the microarray involved in methionine and cysteine biosynthesis were expressed at lower levels when glucose was exhausted, revealing the repression of sulfur assimilation in the absence of glucose (Table 2.3) as occurs in *S. cerevisiae*. One exception is the gene encoding 3'-phosphoadenylylsulfate reductase (*cys-5*). It was induced four-fold under glucose starvation, and its homolog in yeast was also slightly stimulated. Consistent with the inhibition of sulfur utilization, NCU01882.1 (*cys-14*) encodes the mycelial sulfate

Table 2.3 *N. crassa* genes participate in amino acid metabolism.

Ncr ORF [a]	EST ID	Blast Match	Ncr Fold	Sce Homolog	Sce Fold
Amino Acid Biosynthesis					
Transcription Regulation of Amino Acid Biosynthesis					
NCU04050.1	W01D5	Cross-pathway control protein 1	0.38	Gcn4	1.2
NCU04050.1	SM2D1	Cross-pathway control protein 1	0.46	Gcn4	1.2
NCU04050.1	NM8A7	Cross-pathway control protein 1	0.43	Gcn4	1.2
Val, Leu, Ile Biosynthesis					
NCU01666.1	NC5G2	Acetolactate synthase	0.3	Ilv6	0.4
NCU03608.1	SC2F2	Ketol-acid reductoisomerase mitochondrial	0.15	Ilv5	0.23
NCU06232.1	NP2D4	Isopropylmalate dehydrogenase	0.21	Leu2	0.8
NCU03575.1	NC1F12	Isoleucyl-tRNA synthetase	0.41	Ils1	0.27
Met, Cys Biosynthesis					
NCU02657.1	W13G12	Methionine adenosyltransferase	0.28	Sam2	0.35
NCU01985.1	SP1D2	ATP sulfurylase	0.25	Met3	0.35
NCU04077.1	W01C7	Sulfite reductase	0.31	Met10	0.5
NCU02005.1	SC5E11	3' phosphoadenylylsulfate reductase	4	Met16	1.4
NCU06512.1	NC1D10	Methionine synthase	0.96	Met6	0.73
NCU09896.1	NC4E1	Adenylylsulfate kinase	0.74	Met14	0.85
NCU07930.1	SC2C3	S-adenosyl-L-homocysteine hydrolase	0.64	Sah1	0.34
NCU07930.1	W07A2	S-adenosyl-L-homocysteine hydrolase	0.73	Sah1	0.34
NCU09230.1	NC3A10	Cystathionine gamma-lyase	0.87	Str1	0.17
NCU01882.1	NP4D9	Sulfate permease	0.47	NH [b]	
Miscellaneous Amino Acid Biosynthesis					
NCU03935.1	SC2C6	Homoserine dehydrogenase	0.45	Hom6	0.5
NCU02639.1	NC3G2	Argininosuccinate synthase	0.18	Arg1	0.87
NCU08162.1	W17B9	Argininosuccinate lyase	0.22	Arg4	1.1
NCU02274.1	SC6B7	Serine hydroxymethyltransferase	0.44	Shm2	0.37
NCU03500.1	NP6F5	Aminotransferases	0.42	Bna3	0.88
NCU01195.1	NM2B7	NADP-dependent glutamate dehydrogenase	0.76	Gdh1	0.58
Amino Acid Degradation					
NCU00461.1	W01E10	NAD-dependent glutamate dehydrogenase	9.6	Gdh2	4.6
NCU08877.1	SM4H11	Glycine cleavage system H protein	2.9	Gcv3	1.4
NCU01757.1	NM5A10	Asparaginase related protein	9	NH	

[a] The columns in this table are the same as Table 2.1.
[b] NH indicates the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

permease and its expression level was coordinately regulated with the sulfur assimilation pathway (Table 2.3). Another example is the aliphatic amino acid biosynthetic pathway. Valine, leucine and isoleucine, are synthesized from threonine and pyruvate using a common pathway. My data indicate that transcriptions of several genes involved in this pathway were reduced under glucose depletion (Table 2.3).

In contrast to amino acid biosynthetic genes, some genes for amino acid degradation were highly induced during glucose-limited growth. An interesting example was a comparison of the NAD- and NADP-dependent glutamate dehydrogenases. The NAD-dependent glutamate dehydrogenase degrades glutamate to ammonium and alpha-ketoglutarate and was induced ten-fold by glucose starvation (Table 2.3). In contrast, the NADP-dependent glutamate dehydrogenase (*am*), which catalyzes glutamate synthesis, was largely unaffected by glucose level (Table 2.3).

One carbon metabolism is essential for the synthesis of methyl groups, nucleotides and vitamins. It is closely integrated with amino acid metabolism since serine and glycine are the major donors of one carbon units. In *N. crassa* and *S. cerevisiae*, serine is the principle one-carbon donor contributing 5,10-methyl tetrahydrodrofolate (5,10-CH_2-H_4folate) via the reaction catalyzed by serine hydroxymethyltransferase (SHMT) (Jeong and Schirch, 1996; Piper *et al.,* 2000). Glycine catabolism through the glycine cleavage multienzyme complex also provides 5, 10-CH_2-H_4folate to the one-carbon pool (Nagarajan and Storms, 1997**)**. The genes encoding these two enzyme systems have opposite responses to glucose in my microarray experiments. Transcription of SHMT was repressed, but the gene encoding

glycine cleavage H protein was induced when the cells were subjected to glucose limitation (Table 2.3). In yeast, it has been reported that inhibition of SHMT lowered the cytoplasmic 5, 10-CH_2-H_4folate level and the low level of 5,10-CH_2-H_4folate stimulates the expression of the glycine cleavage system to supplement the reduced level of 5,10-CH_2-H_4folate (Piper *et al.,* 2000). Therefore, the glucose regulation of the glycine cleavage system may be an indirect response to the down-regulation of SHMT.

Identification of putative glucose transporters

Transport of glucose into cells is the first and limiting step of glucose regulation. Two glucose transport systems have been characterized in *N. crassa*, a constitutive low-affinity transport system and a glucose-repressible high-affinity transport system (Scarborough, 1970; Schneider and Wiley, 1971). Thirty-three hexose transporter homologs have been identified in the *N. crassa* genome sequence, representing one of the largest multigene families. Phylogenetic analyses indicated that the relationship between *S. cerevisiae* and *N. crassa* hexose transporter paralogs is poor and no clear orthologs for comparison of gene expression are present (Galagan *et al.,* 2003). Four predicted glucose transporter homologs were represented on the microarray. Three of these were found to be negatively regulated by glucose (Table 2.4). One of them, NCU10021.1 was highly expressed upon glucose depletion in microarray experiment and was further examined by Northern analysis (Figure 2.3). NCU10021.1 has been demonstrated to encode a high-affinity glucose transporter in *N. crassa* (Chapter IV). Interestingly, NCU06358.1, encoding another hexose transporter homolog, was marginally lower under glucose starvation conditions. The decline in expression

observed in the microarray experiments was confirmed by Northern blot analyses (Figure 2.3). These data suggest that NCU06358.1 may encode a low affinity transporter that is more highly expressed in glucose-rich medium (Figure 2.3). A monocarboxylate permease homolog has elevated transcription in the absence of glucose (Table 2.4), consistent with a need to transport pyruvate and/or acetate into the cell or mitochondrion under glucose-limited conditions.

Glucose regulation of genes for alternate carbon sources and sporulation

After glucose was depleted, expression of genes for utilization of alternative carbon sources including ethanol (alcohol dehydrogenase, aldehyde dehydrogenase), glycerol (glycerol kinase), and glycogen (glucan 1,4-alpha-glucosidase) became elevated (Table 2.4). *grg-1* (*glucose repressible gene*) encodes a stress protein whose regulation by glucose has been well-characterized in *N. crassa* (Wang *et al.,* 1994). *grg-1* transcript levels were elevated by an average of 9-fold and this increase was supported by Northern blot analysis (Figure 2.3). *grg-1* is also known to be induced during asexual sporulation, and several other developmentally regulated genes were found to respond to glucose. The *eas* gene is expressed early in conidiation and displayed a positive response to glucose starvation in minimal medium (Table 2.4) (Figure 2.3). However, *eas* expression was not observed in medium containing peptone, confirms that nutritional regulation of *eas* is subject to multiple levels of regulation (Figure 2.3) (Bell-Pedersen *et al.,* 1996). Other developmentally regulated genes, including *con-6, con-10*, displayed an elevated expression upon two hours glucose starvation (Table 2.4). Thus, many developmentally regulated genes appear to be also subject to glucose regulation.

Table 2.4 *N. crassa* genes participate in transport, alternative carbon utilization, carbon repression and development.

Ncr ORF [a]	EST ID	Blast Match	Ncr Fold	Sce Homolog	Sce Fold
Transport					
NCU08114.1	SM4A3	Hexose transporter	7.5		
NCU00988.1	NM5H7	Hexose transporter	3.98		
NCU10021.1	NM4B6	Hexose transporter	3.08		
NCU10021.1	SP1A4	Hexose transporter	10.6		
NCU06358.1	NP4E6	Hexose transporter	0.88		
NCU05089.1	NP3E1	Monocarboxylate permease	2.6	Mch5	1.8
Glycerol, Glycogen and Ethanol Utilization					
NCU06005.1	NM1C7	Glycerol kinase	2.22	Gut1	3
NCU06005.1	W08B12	Glycerol kinase	3.11	Gut1	3
NCU01517.1	NM6B8	Glucan 1,4-alpha-glucosidase	14.3	Sga1	1.8
NCU01754.1	NC4C1	Alcohol dehydrogenase	3.79	Adh2	0.6
NCU01754.1	NC5E1	Alcohol dehydrogenase	3.88	Adh2	0.6
NCU01754.1	SM2D6	Alcohol dehydrogenase	7.7	Adh2	0.6
NCU09266.1	SP3B11	Aldehyde dehydrogenase	7.45	Ald2	4.8
Carbon Repression/Development					
NCU03753.1	SP6C9	Glucose-repressible gene protein (grg-1)	10.3	NH [b]	
NCU03753.1	NM6B6	Glucose-repressible gene protein (grg-1)	8.78	NH	
NCU03753.1	NP5G7	Glucose-repressible gene protein (grg-1)	14.3	NH	
NCU03753.1	CCG-1	Glucose-repressible gene protein (grg-1)	3.59	NH	
NCU08457.1	CCG-2	Hydrophobin (eas)	1.52	NH	
NCU08769.1	CON-6	Conidiation specific protein-6	3.5	NH	
NCU08769.1	pCON-6	Conidiation specific protein-6	1.34	NH	
NCU07325.1	CON-10	Conidiation specific protein-10	2.28	NH	
NCU07325.1	pCON 10	Conidiation specific protein-10	1.56	NH	

[a] The columns in this table are the same as Table 2.1.
[b] NH indicates that the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

Cell growth and signal transduction

Genes involved in cell growth includes genes encoding translation initiation factors, ribosomal proteins and cytoskeleton components. Actin is usually thought of as a housekeeping gene that is constitutively expressed. For example, actin transcript levels do not change appreciably during the diauxic shift in *S. cerevisiae* (DeRisi *et al.,* 1997). However, it has long been observed in Northern blot experiments with *N. crassa* that actin mRNA levels can vary depending on growth condition or developmental stage. Here I found that actin transcript levels declined upon glucose deprivation as determined by both the microarray (approximately three-fold) and Northern blot experiments (Table 2.5) (Figure 2.3). Transcripts for a number of other cytoskeleton proteins were also down-regulated (Table 2.5). Previous studies indicated transcription control plays a major role in the regulation of ribosome genes in response to carbon availability in *S. cerevisiae* and *N. crassa* (Morrow *et al*., 1993; Cujec and Tyler, 1996). The repression of the general transcription machinery and induction of vacuolar and ubiquitin-mediated protein degradation (Table 2.5) indicated a coordinated mechanism for regulation of protein metabolism, reflecting the need for catabolism to provide the fuel for growth in the absence of glucose. The down-regulation of genes involved in cell growth is similar to previous observations that the levels of 40S rRNA and three ribosome protein mRNAs rapidly decreased within the first two hours of a shift from sucrose to glycerol medium (Cujec and Tyler, 1996).

Transcript levels of the *N. crassa* homolog of the *S. cerevisiae SLT2* MAP kinase declined upon shift to glucose-free medium (Table 2.5). In *S. cerevisiae*, five MAP

Table 2.5 *N. crassa* genes participate in general biosynthesis and cellular communication.

Ncr ORF [a]	EST ID	Blast Match	Ncr Fold	Sce Homolog	Sce Fold
Cytoskeleton Synthesis					
NCU09468.1	NP6G3	Alpha-tubulin	0.49	Tub1	0.24
NCU00202.1	W13D12	Coronin G-beta repeat	0.34	Crn1	0.6
NCU01204.1	NC4E9	Tropomyosin	0.43	Tpm2	0.57
NCU01587.1	NM4B4	Cofilin	0.47	Cof1	0.55
NCU04173.1	W17E2	Actin	0.34	Act1	0.97
NCU04173.1	ACTIN	Actin	0.36	Act1	0.97
NCU04173.1	ACTIN	Actin	0.35	Act1	0.97
NCU04173.1	ACTIN	Actin	0.31	Act1	0.97
Protein Synthesis					
NCU00258.1	SC5A9	40S ribosomal protein	0.42	Rps7a	0.23
NCU08389.1	W10G5	Rat L18a (ribosome protein)	0.24	Rpl20b	0.19
NCU02208.1	SP6G12	Translation initiation factor eIF3	0.43	Prt1	0.3
NCU03826.1	NC1A6	Elongation factor 1 (EF-1)	0.4	Cam1	0.18
NCU03826.1	NM6D9	Elongation factor 1 (EF-1)	0.25	Cam1	0.18
NCU06035.1	NC1A10	Translation elongation factor eEF-1	0.44	Efb1	0.2
NCU07420.1	NP5E5	Translation initiation factor eIF-4A	0.37	Tif1	0.2
NCU07420.1	NC1D6	Translation initiation factor eIF-4A	0.41	Tif1	0.2
Protein Degradation					
NCU00477.1	NM1E4	Carboxypeptidase	3.9	Prc1	3.8
NCU10046.1	NM3E1	Ubiquitin conjugating enzyme	2.3	Ubc8	5
NCU04274.1	SC3F4	Transport (ubiquitin protein degradation)	2.5	Sts1	0.85
Cellular Communication					
NCU09842.1	NC4D2	MAP kinase	0.44	Slt2	1.1
NCU09842.1	SC2D11	MAP kinase	0.25	Slt2	1.1
NCU00443.1	SC5C8	Ran(G-protein)/spi1 binding protein	0.44	Yrb1	0.4
NCU00443.1	W07A9	Ran(G-protein)/spi1 binding protein	0.43	Yrb1	0.4
NCU08923.1	NM5F11	DNA-binding protein	0.31	Gls2	0.36
NCU01613.1	SP1F10	Shk1 kinase-binding protein 1	14	Hsl7	0.7
NCU02463.1	SM2A6	receptor-associated protein	3.2	Ynl305c	2.2
NCU00625.1	NM4H10	UVSB PI-3 kinase	3.2	Srp101	0.76
NCU02801.1	W08E3	Benzodiazepine receptor	3.4	NH [b]	
NCU02469.1	SP1D5	Protein kinase-like protein	9.4	NH	
NCU10028.1	W01B11	receptor-associated protein	2.7	NH	
NCU03967.1	W07H6	VVD	2.8	NH	

[a] The columns in this table are the same as Table 2.1.
[b] NH indicates that the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

kinase pathways have been well-studied (Gancedo, 1998). SLT2 is involved in the cell wall integrity signaling pathway and activation of SLT2 protein induces expression of genes encoding cell wall proteins and proteins involved in cell wall biogenesis (Jung and Levin, 1999). The reduced transcription of SLT2 homolog in my experiment is consistent with a reduction in growth rate in response to glucose starvation. Two *S. cerevisiae Yrb1* and *Gis2* homologs are also down-regulated (Table 2.5). The Ran-binding protein YRB1 is involved in cell cycle regulated protein turnover and depletion of YRB1 results in decreased progression through mitosis (Baumer *et al.,* 2000). Gis2 is a DNA-binding protein thought to inhibit the cAMP pathway in *S. cerevisiae* and is capable of suppressing the mating defect of Ras1/Ras1 diploids of *Schizosaccharomyces pombe* (Balciunas and Ronne, 1999). Therefore, the *N. crassa* homolog might be involved in Ras and cAMP signaling to regulate pathways for cellular growth and/or regulation of the switch from glycolytic to gluconeogenic metabolism. An *Hsl7* ortholog was highly induced by the shift to glucose starvation (Table 2.5). HSL7 is a protein methyltransferase that interacts with both Ste20 and Swe1 to negatively regulate the filamentation/mating MAP kinase pathway and the cell cycle (McMillan *et al.,* 1999). Together, these data reflect a decline in transcripts for genes that are activators of cell growth and an increase in transcripts for genes that act as inhibitors of growth and cell cycle progression following glucose starvation.

Finally, the *vvd* gene plays an important role in light regulation of gene expression in *N. crassa* and *vvd* mutants are defective in down regulation of light-inducible genes (adaptation), such as *con-10* (Bailey-Shrode *et al.,* 2001). Elevated

expression of *vvd* in response to glucose downshift suggests that light-regulated genes may adapt more efficiently to light in the absence of glucose.

DISCUSSION

Response to carbon downshift

Microarray analysis has recently been applied to filamentous fungi, such as *N. crassa* and *T. reesei* (Chambergo *et al.,* 2002; Lewis *et al.,* 2002). Central metabolism has been well-characterized in *S. cerevisiae* and has been the subject of physiological studies in filamentous ascomycete fungi. I chose to use a two hour shift to glucose-free medium to examine central metabolism in *N. crassa* because these conditions lead to maximum induction of sugar transport activities, demonstrating that this period of time is sufficient to observe gene regulation in response to carbon source (Scarborough, 1970; Schneider and Wiley, 1971; Madi *et al.,* 1997). Microarray analysis of these conditions supports the view that *N. crassa* carries out alcoholic fermentation and respiration simultaneously in the presence of glucose and the downshift to carbon starvation leads to a shutdown of genes for glycolysis and elevated expression of genes for gluconeogenesis. As a whole, the TCA cycle appears to be weakly regulated by glucose, although some genes of the TCA cycle are coordinately regulated with the genes of the glyoxylate cycle.

Previous studies demonstrated similar transcription responses of the genes encoding malate synthase, isocitrate lyase and pyruvate decarboxylase (Table 2.6). *N. crassa* has been the subject of physiological studies for several decades and enzyme

activities have been measured for many of the steps of central metabolism (Table 2.6). Activities for enzymes in glycolysis, the TCA cycle, the glyoxylate cycle, the beta-oxidation of fatty acids, and NAD- and NADP-dependent glutamate dehydrogenases are regulated very similarly to the transcripts for the corresponding genes as determined by this study (Table 2.6). The results from Northern blot analysis of the expression patterns of genes for two glucose transporter homologs, phosphoenolpyruvate carboxykinase, actin, *grg-1* and *eas* also confirmed my microarray data (Figure 2.3).

The growth conditions tested here measure the changes on the transcription when cells are transferred from medium containing glucose to medium lacking glucose, while many of the previously published studies of transcript levels or enzyme activities measure the response to medium shifts to secondary carbon sources or adapted growth on secondary carbon sources. The correlation of the results between my data and this previously published work suggests that, in general, these different growth parameters do not result in fundamentally different responses. However, several differences between transcript and enzyme activity levels in the TCA cycle may be due to differences in growth conditions or the period of adaptation (Table 2.6). Although the observed transcriptional responses of genes for citrate synthase, NAD-isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, succinate dehydrogenase and fumarase are similar to the measured changes in enzyme activities, transcripts for two of the TCA cycle genes respond differently from their enzyme activities. Transcripts encoding malate dehydrogenase increase (approximately 4-fold) in the microarray experiment, but

Table 2.6 *N. crassa* genes with previous measured enzyme activities and transcription level.

Ncr ORF [a]	EST ID	Blast Match	Ncr Fold	Enzyme Fold [b]	Previous Validation [c]
Glycolysis/Gluconeogenesis/fermentation					
NCU07550.1	SC1G8	Triosephosphate isomerase	0.43	0.048 [e]	
NCU07550.1	NC2E4	Triosephosphate isomerase	0.44	0.048 [e]	
NCU09873.1	NM5C3	Phosphoenolpyruvate carboxykinase	33	28 [j]	
NCU09873.1	SM3H9	Phosphoenolpyruvate carboxykinase	29	29 [j]	
NCU06836.1		Acetyl-CoA synthetase	NP[i]		Yes [g]
NCU02193.1	NC3D2	Pyruvate decarboxylase	0.19		Yes [h]
Citrate Cycle (TCA Cycle)					
NCU01692.1	NM6F5	Citrate synthase	4.5	3.8 [j]	
NCU00775.1	SC1C8	NAD-isocitrate dehydrogenase	1.2	1.7 [l]	
NCU02438.1	NM1B4	Alpha-ketoglutarate dehydrogenase	0.8	0.76 [j]	
NCU08471.1	W13H10	Succinyl-CoA ligase	0.3	1.0 [j]	
NCU08336.1	NC1F7	Succinate dehydrogenase	3.5	5.6 [f]	
NCU00959.1	NM7H6	Succinate dehydrogenase	5.3	5.6 [f]	
NCU03031.1	SC5F1	Succinate dehydrogenase	4.2	5.6 [f]	
NCU10008.1	NM1B3	Fumarase	1.3	2.1 [d]	
NCU04899.1	W08E4	Malate dehydrogenase	4.5	0.63 [k]	
NCU04899.1	W08B12	Malate dehydrogenase	3.1	0.63 [k]	
Glyoxylate Cycle					
NCU02481.1	NM3F4	Isocitrate lyase	2.9	48 [f]	Yes [g]
NCU04230.1	NP3E9	Isocitrate lyase	11	49 [f]	
NCU03857.1	W17F2	NADP-isocitrate dehydrogenase	2.8	4.4 [l]	
NCU06211.1	NP2E7	Malate dehydrogenase	6	3.9 [j]	
NCU10007.1		Malate synthase	NP[i]	40 [f]	Yes [g]
Amino Acid Metabolism					
NCU01195.1	NM2B7	NADP-glutamate dehydrogenase	0.76	0.51 [j]	
NCU00461.1	W01E10	NAD-glutamate dehydrogenase	9.6	38 [j]	
Fatty Acid Degradation					
NCU08828.1	NP2E8	hydratase-dehydrogenase	5.5	14 [d]	
NCU08828.1	NM4H8	hydratase-dehydrogenase	5.1	14 [d]	
NCU04796.1	NM5E11	Peroxisomal 3-ketoacyl-CoA thiolase	4.5	22 [d]	

Table 2.6 (continued)
[a] column Ncr ORF, EST ID, Blast Match, Ncr Fold are the same as Table 2.1.
[b] Enzyme Fold is the ratio of enzyme activity of cell grown in glucose poor medium to glucose grown cell
[c] Previous Validation indicate similar transcription regulation was observed by published reports
[d] Kionka and Kunau, 1985
[e] Tom *et al.,* 1978
[f] Flavell and Woodward, 1970a
[g] Bibbins *et al.,* 2002
[h] Alvarez *et al.,* 1993
[i] Not Present on Array
[j] Flavell and Fincham, 1968
[k] Benveniste and Munkres, 1970
[l] Kobr *et al.,* 1965

enzyme activities display a decline (1.5-fold) (Table 2.6). I also found that transcript levels for succinyl-CoA ligase decreased, while enzyme activity did not change. These differences do not affect the main conclusion that the overall capacity of the TCA cycle does not change significantly during the shift to a secondary carbon source. However, I found that succinate dehydrogenase and malate dehydrogenase transcript levels are co-regulated with the genes for the glyoxylate cycle and phosphoenoylpyruvate carboxykinase. A plausible explanation is that cells are preparing to utilize storage compounds, such as lipids and amino acids, and these enzyme activities are needed to maximize production of oxaloacetate for gluconeogenesis using both the glyoxylate cycle and part of the TCA cycle (Figure 2.2).

Consistent with this view is the coordinated regulation of lipid and amino acid metabolism where biosynthetic gene transcripts decline and degradative gene transcripts increase. One particular example is the coordinated increase in transcripts of the genes for beta-oxidation of fatty acids and the glyoxylate cycle, whose protein products are co-localized to the glyoxysome. This transcriptional response is similar to the increase in enzyme activities observed when *N. crassa* is shifted to acetate or oleate as a sole carbon source (Flavell and Woodward, 1971a; Kionka and Kunau, 1985). Thus, the immediate physiological response to abrupt glucose starvation resembles the response of cells downshifted to acetate or lipid as carbon sources.

In *N. crassa,* a downshift to carbon starvation acts as a signal to induce asexual sporulation (conidia formation). In previous studies, differences in the activities of a number of enzymes of central metabolism were observed in comparisons of mycelia

cultures and cultures undergoing asexual sporulation. For example, succinate dehydrogenase is less abundant in mature conidia than in mycelia but has maximal activity at the time of conidiophore initiation (Turian and Bianchi, 1972). Therefore, the earliest stages of induction of conidiation may be reflected in my observation of elevated transcript levels for succinate dehydrogenase. *fluffy*, a mutant that blocks conidia formation, was reported to possess lower isocitrate lyase and cytosolic malate dehydrogenase activity on acetate medium than the wild type (Turian and Bianchi, 1972). In general, culture conditions that favor fermentation favor mycelia growth, while culture conditions unfavorable to fermentation promote conidiation. This observation suggested that the relative balance of fermentation and respiration regulate conidiation in *N. crassa* (Weiss and Turian, 1966). The induction of developmentally regulated genes, such as *eas* (Figure 2.3) (Table 2.4), *con-10* and *con-6* (Table 2.4) might be interpreted to be the result of very early signaling in development. However, my interpretation is that regulation of these genes is simply due to their regulation by glucose as was previously noted for *con-10* and *eas* (Kaldenhoff and Russo, 1993; Bell-Pedersen *et al.,* 1996; Lee and Ebbole, 1998a) and the issue of whether a shift in central metabolism is a signal for conidiation remains to be addressed.

Glycolysis and fermentation of glucose

In the presence of glucose, *N. crassa* simultaneously carries out respiration and fermentation as shown by expression of genes for these processes and previous measurements of enzyme activities and respiration rates (Flavell and Fincham, 1968; Flavell and Woodward, 1970a; Flavell and Woodward, 1971a; Colvin *et al.,* 1973a;

Colvin *et al.,* 1973b). The higher level of both pyruvate dehydrogenase and pyruvate decarboxylase transcripts in glucose containing medium suggests that pyruvate is being channeled into both the TCA cycle and fermentation. One way to generate acetyl-CoA for oxidation is via the pyruvate bypass (conversion of ethanol or acetate to acetyl-CoA) without using the pyruvate dehydrogenase complex. The lower levels of transcripts for alcohol dehydrogenase (ADH2) and aldehyde dehydrogenase (ALD2) in the presence of glucose supports the view that acetyl-CoA is primarily generated by pyruvate dehydrogenase and not by the pyruvate bypass. Previous measurements of acetyl-CoA synthetase activity are consistent with this conclusion (Flavell and Woodward, 1970a). Although adh1, adh2, and ald2 homologs were identified as the best bi-directional hits with *S. cerevisiae*, I can not be certain that they are the key enzymes in *N. crassa* for carrying out the steps as illustrated in Figure 2.2. Therefore, some caution is required in the interpretation of the pathways until mutational studies define the roles of these genes.

The low level of activity of the pyruvate bypass in the presence of glucose is expected to allow efficient accumulation of ethanol, and accumulation of ethanol in *N. crassa* cultures has been well documented. In medium with 2% glucose, *N. crassa* accumulated ethanol in culture filtrates up to 0.4% (wt/vol) (Colvin *et al.,* 1973b). With 10% glucose, the ethanol concentration can accumulate to 2% and the efficiency of conversion of glucose to ethanol approached 100% as stationary phase was reached (Colvin *et al.,* 1973b). After downshift, the reduced transcript levels for pyruvate dehydrogenase and increased transcript levels for alcohol dehydrogenase and aldehyde dehydrogenase suggests that *N. crassa* is to efficiently utilize ethanol as a carbon and

energy source. This is consistent with previous findings that ethanol accumulation in *N. crassa* reached a maximum level upon glucose exhaustion and subsequently declined to zero (Colvin *et al.,* 1973b).

Comparison of the responses of *N. crassa* and *S. cerevisiae* transcript levels indicates that these two fungi share similar responses to glucose in that fermentation is active and the glyoxylate cycle is repressed by glucose. The major difference is that all steps of the TCA cycle are repressed by glucose in *S. cerevisiae* but several of these genes are not repressed in *N. crassa* (Figure 2.2). This is consistent with the fact that *S. cerevisiae* primarily generates energy through fermentation in glucose-rich medium whereas *N. crassa* clearly generates energy through both fermentation and the TCA cycle/respiration.

T. reesei and *N. crassa* are both saprophytes deriving energy from degradation of plant material. The *N. crassa* genome sequence reveals a large number of extracellular plant cell wall degrading enzymes that allow utilization of cellulose and other plant cell wall materials (Galagan *et al.,* 2003). Recently, transcript levels for a number of genes of central metabolism in *T. reesei* were examined (Chambergo *et al.,* 2002). It was concluded that *T. reesei* primarily utilizes glucose via respiration and carries out a low rate of conversion of pyruvate to acetate (Chambergo *et al.,* 2002). This observation is important because production of ethanol involves oxidation of NADH to NAD^+, while production of acetate further uses NAD^+ to produce NADH. This suggests that the rate of glycolysis (requiring NAD^+) and respiration (generating NAD^+) are balanced to maintain the NAD^+/NADH ratio in *T. reesei*. In contrast, *N. crassa* utilizes glucose in a

manner in which the rate of glycolysis exceeds the rate at which the products of glycolysis are utilized by the TCA cycle and respiration. In this view, fermentation to ethanol rather than acetate is required to generate NAD^+ to support the high rate of glycolysis. *N. crassa* is well known for its rapid growth rate. Hyphae can extend up to 12 cm/day on solid medium and the doubling time in liquid culture is approximately 2 h. Energy demands to support this high growth rate may require fermentation as well as respiration. Another possibility is that, in nature, removal of glucose from a glucose-rich environment and production of ethanol is used as a strategy for competition with other microbes.

The fact that *N. crassa* ferments glucose to ethanol suggests that it may have advantages over yeast or other filamentous fungi for bioethanol production. It is significant that *N. crassa* already possesses a wide range of enzymes to degrade plant cell walls and could be used to ferment complex substrates. Improving the ability of *S. cerevisiae* to degrade plant cell wall material is possible, but overall, yeasts are not well adapted to using complex lignocellulosic materials (Walfridsson *et al.,* 1995; Zaldivar *et al.,* 2002). Although other filamentous fungi, such as *T. reesei,* are efficient degraders of plant cell wall materials, unlike *N. crassa*, they have not evolved to produce ethanol as a fermentation product. Thus, *N. crassa* may be an excellent model system to explore pathway engineering to optimize bioethanol production by filamentous fungi.

This study adds to the tremendous wealth of information describing the behavior of genes in pathways for central metabolism in *N. crassa*. It is important to note that past studies include analysis of wild-type and key mutant strains affected in central

metabolism and respiration (Flavell and Woodward, 1970a; Flavell and Woodward, 1971a; Flavell and Woodward, 1971b; Kolvin *et al.,* 1973a). Thus, much of the information to help guide metabolic engineering of *N. crassa* for efficient generation of ethanol from renewable resources is available. For example, it is already clear from mutant analysis that reducing respiration rates does not lead to increased fermentation in *N. crassa* (Flavell and Woodward, 1971b). However, manipulation of regulatory genes for glycolytic enzyme production might be used for improving ethanol production. The analysis also suggests that strains deficient in utilization of ethanol (aldehyde dehydrogenase and acetyl-CoA synthetase mutants) would be candidates for enhanced ethanol-accumulation. Further improvement of plant cell wall utilization could be achieved more easily in *N. crassa* than *S. cerevisiae* by increasing expression of endogenous extracellular cell wall degrading enzymes or by addition of new genes for such enzymes from related fungi, such as *T. reesei*.

CHAPTER III

TRANSCRIPTIONAL PROFILING OF *NEUROSPORA CRASSA* MUTANTS AFFECTING SUGAR SENSING AND ASEXUAL DEVELOPMENT

OVERVIEW

The *rco-3* gene was proposed to encode a sugar sensor that regulates sugar transport and conidiation in *N. crassa* (Madi *et al.,* 1997). A 2-deoxyglucose resistant mutant (*dgr-1*) phenotypically resembles *rco-3* and may be part of the *rco-3* signaling pathway. To further investigate the relationship between *rco-3* and *dgr-1* and to help assess the extent to which they may be in a common regulatory cascade, the transcriptional response to glucose status in *rco-3* and *dgr-1* mutants was examined via microarray technology. The two mutants display similar expression patterns for most of the genes on the microarray supporting a close functional relationship between them. The study indicates that *rco-3* and *dgr-1* induce conidiation at least partially independent of sugar transport defects. *rco-3* and *dgr-1* are required for appropriate regulation of genes involved in the TCA cycle, the glyoxylate cycle and gluconeogenesis, supporting the view that *rco-3* and *dgr-1* appear to control multiple pathways to regulate different aspects of cellular physiology.

INTRODUCTION

Nutrient availability is the major factor controlling growth and development among competing microorganisms in an environment with limited resources. Glucose is

the primary carbon and energy source for most eukaryotic cells. In addition, glucose serves important hormone-like roles as a primary messenger to regulate cell growth, metabolism and development (Rolland *et al.,* 2001). In *Saccharomyces cerevisiae*, glucose stimulates its own use by inducing transcription of genes involved in glucose catabolism while repressing genes encoding enzymes for utilization of alternative carbon sources and gluconeogenesis. This regulatory phenomenon is well-known as carbon catabolite repression (Gancedo, 1998).

Sensing the existence of extracellular sugar is the first step of glucose regulatory machinery. Sugar sensing and signaling mechanisms are extensively studied in eukaryotic cells, especially in *S. cerevisiae* (D'Souza and Heitman, 2001; Rolland *et al.,* 2001; Rolland *et al.,* 2002). Four distinct signaling cascades have been proposed for glucose regulatory machinery in budding yeast (Rolland *et al.,* 2001). The hexokinase (HXK2) dependent carbon repression pathway is responsible for long term glucose repression and MIG1 is the transcriptional repressor. The second pathways posses two sugar sensors (SNF3 and RGT2) and mainly control the expression of functional hexose carriers in which RGT1 functions as repressor and activator. Glucose activation of cAMP pathways requires glucose phosphorylation and activation of a G-protein coupled receptor (GPCR) system. The GPCR system, including Gpr1 and Gpa2 genes, regulates downstream adenylate cyclase and protein kinase A to activate glycolytic genes and repress genes in gluconeogenesis (Rolland *et al.,* 2001). The fourth pathway is not well defined and appears to utilize intermediate metabolites of glycolysis as metabolic messengers.

Asexual sporulation in filamentous fungi often serves as the primary means of disease dispersal. This includes fungi that are causal agents of human and plant disease. Therefore, understanding the mechanisms governing asexual sporulation may help to control fungal disease. *N. crassa* is a model system for studies of asexual development (macroconidiation) in filamentous fungi (Springer, 1993; Ebbole, 1996). Several environmental stimuli including glucose deprivation can induce conidiation in *N. crassa*. The *rco-3* mutant was originally isolated due to elevated expression of a conidiation specific gene (*con-10*) (Madi *et al.,* 1994). Subsequent cloning and characterization showed that *rco-3* is involved in regulation of conidiation, sugar transport and carbon repression in *N. crassa* (Madi *et al.,* 1997). The *rco-3* gene has most sequence similarity to the sugar transporter superfamily in *S. cerevisiae* and was proposed to encode a sugar sensor in *N. crassa* (Madi *et al.,* 1997). Therefore *rco-3* may provide a link between glucose sensing and conidiation in *N. crassa*.

Previous study prompted us to hypothesize that RCO-3 is the counterpart of SNF3 and RGT2, two glucose sensors in *S. cerevisiae* (Madi *et al.,* 1997). SNF3 is necessary for the induction of hexose transporters when glucose levels are low whereas RGT2 is required for induction at high glucose concentration (Özcan *et al.,* 1996; Özcan and Johnston, 1999). Rgt1 (restore glucose transport 1), originally isolated as a suppressor of *snf3*, encodes a transcription repressor and activator of SNF3/RGT2 pathways (Marshall-Carlson *et al.,* 1991; Özcan and Johnston, 1999). RGT1 is regulated by a multiprotein complex (SCF^{Grr1}) through the protein ubiquitin proteolysis pathway (Li and Johnston, 1997). In addition, MTH1 and STD1 interact with the hydrophilic C-

terminal tails of SNF3 and RGT2 and might be involved in transmission of SNF3/RGT2 signal (Schmidt *et al.,* 1999; Lafuente *et al.,* 2000).

Compared to the wealth of information in *S. cerevisiae*, the mechanisms by which filamentous fungi sense glucose and regulate internal physiological response is less well understood. *rco-3* has pleiotropic effects on sugar transport, carbon repression and conidiation (Madi *et al.,* 1997). Therefore, understanding the *rco-3* signaling pathway and its downstream targets will provide a basis to elucidate the sugar sensing pathways and how glucose limitation triggers conidiation in filamentous fungi. 2-deoxyglucose is a toxic sugar analog that inhibits growth of fungal cells (Moore, 1981). Previously isolated 2-deoxyglucose resistant mutants, *dgr-1* and *dgr-3*, have phenotypes similar to *rco-3* (Allen *et al.,* 1989), and *rco-3* is allelic with *dgr-3*. Therefore, *dgr-1* may be involved in the putative *rco-3* signaling pathway.

Transcription regulation plays an important role in allowing cells to adapt to different nutrient sources. Previous studies demonstrate that glucose regulation mainly takes place at the transcriptional level in fungi (Gancedo, 1998; Zeeman *et al.,* 2000; Chambergo *et al.,* 2002; Chapter II). Characterization of the genes regulated by *rco-3* and *dgr-1* will provide insights to elucidate the functions of these genes and their relationship to one another. A cDNA microarray containing 1363 *N. crassa* genes was used to examine the gene expression profile of *rco-3* and *dgr-1* mutants under conditions of abundance and depletion of glucose. I found that *rco-3* and *dgr-1* mutants have similar transcript profiles supporting the view that they participate in a common signaling pathway. The pathway involving *rco-3* and *dgr-1* affects expression of genes

for glucose catabolism, lipid metabolism, amino acid metabolism as well as general biosynthesis and degradation in *N. crassa*. In addition, *rco-3* and *dgr-1* are required for appropriate regulation of the TCA cycle, the glyoxylate cycle and gluconeogenesis. Comparing transcripts differentially expressed in *rco-3* and *dgr-1* with previous identified glucose-regulated genes suggests that *rco-3* and *dgr-1* mutants are generally derepressed for carbon catabolite repression and conidiation. However, *rco-3* and *dgr-1* induce conidiation at least partially independent of glucose starvation effects.

MATERIALS AND METHODS

Strains and growth conditions

Unless indicated, strains used in this study are obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas, Kansas City, Kansas, 66160-7420. Strain Oak Ridge 74OR23-1A (FGSC no. 987) is the wild type strain. *rco-3* (null ripping mutant generated in our laboratory) and *dgr-1* (FGSC no. 4326) were used in this study.

Cultures were grown in Vogel's minimal medium (Davis and De Serres, 1970) supplemented with glucose as described previously (Madi *et al.,* 1997). Conidia were collected with sterile water through Miracloth (Calbiochem) from a 50 ml solid medium culture after six days of growth. Conidia were counted with a hemocytometer (Hausser Scientific) and inoculated at 10^6 conidia/ml into 50 ml of liquid minimum medium containing 2% peptone and 2% glucose. Liquid cultures were grown approximately 17-18 hours on a rotary shaker (200 rpm) at 34°C. The mycelium was harvested by vacuum filtration on sterile Whatman filter paper and cut into two pieces with a sterile razor

blade to yield mycelium pads of approximately equal size. Each mycelia pad was inoculated into either fresh 2% glucose-supplemented minimal medium (glucose repression) or the same medium lacking glucose (glucose derepression). The inoculated flasks were vigorously shaken to disperse the cells and incubated for two hours at 34°C. Cultures were harvested by filtration and frozen in liquid nitrogen prior to RNA extraction. In order to reduce the variation arising from an individual growth experiment, mycelium was harvested from five independent cultures using the same growth conditions and combined for RNA extraction. Resistance to 2-deoxyglucose (Sigma) and sorbose were examined on solid Vogel medium as previously reported (Madi *et al.,* 1997).

Nucleic acid isolation, hybridization and sequencing

Genomic DNA was isolated as previously described (Vollmer and Yanofsky, 1986). RNA extraction and Northern blot hybridization were performed following the protocol described previously (Madi *et al.,* 1994). Northern blot analyses use RNA preparation from one growth culture, whereas microarray experiments use the combined RNA preparation from multiple cultures. mRNA was isolated from total RNA using a polyATtract mRNA isolation system IV kit (Promega). DNA probes for NCU10021.1 (cDNA clone SP1A4), NCU06358.1 (cDNA clone NP4E6), NCU09873.1 (phosphoenoylpyruvate carboxykinase), and *grg-1* were obtained from the corresponding sequence-verified EST clones by PCR. RNA quality and loading were verified by visual inspection of rRNA band intensity and hybridization with rDNA probes. Sequencing of cDNA clones was performed using the Prism dideoxy sequencing

kit (Applied Biosystem) with T3 and T7 primers in the Gene Technology Laboratory (Texas A&M University).

Microarray construction and hybridization

EST (expressed sequence tag) libraries representing different stages of the *N. crassa* life cycle (germinated conidia, mycelia and sexual stage) were provided by the *Neurospora* genome project at the University of New Mexico (Nelson *et al.,* 1997). 1764 EST sequences from three EST libraries were assembled using STACKPACK™ software (Electric Genetics). BLAST analysis of the EST sequences against the newly released *N. crassa* genomic sequence from the WhiteHead institute indicated that 1363 unique genes were represented, accounting for approximately 14% of all *N. crassa* genes. The difference between the number of ESTs and the unigene set is due to multiple representations of genes in our EST collection, which also provide controls to examine the reproducibility of microarray hybridization with different cDNA clone PCR products representing the same gene. cDNA inserts were amplified by PCR in 96-well format using universal T3 and T7 primers by our laboratory and Dr. Deborah Bell-Pedersen's laboratory. PCR products were verified by agarose gel electrophoresis. Purified PCR products were dissolved in 3 X SSC and printed on poly-lysine slide in the Laboratory for Functional Genomics in the Department of Biology (Lewis *et al.,* 2002). Genes from other organisms - *Arabidopsis thaliana pap-1* and the *verK* gene from *Aspergillus nidulans*, were placed on the microarray slide as negative controls (Lewis *et al.,* 2002).

The two channel (Cy3 and Cy5) labeling system, which allows simultaneous comparison of gene expression patterns between two samples (DeRisi *et al.,* 1997), was

used. mRNA (300 ng) from each sample was reverse transcribed and hybridized to the microarray following the two-step protocol from 3DNA^{TM} Submicro EX Expression Array Detection Kit (Genisphere). The TIFF (Tag Image File Format) images were obtained using an Affymatrix 426^{TM} Array Scanner (Affymatrix). Due to photobleaching of the fluorescent dyes, especially Cy5, the scanner laser power and photo-multiple tube (PMT) settings were adjusted to balance the signals in the two channels. The TIFF images of each channel were imported into ScanAlyze to obtain raw signal intensities for each spot (ScanAlyze was written by Michael Eisen)

Expression data analysis

Three replicate microarray experiments of *rco-3* and *dgr-1* strains under glucose repression and derepression condition were conducted. Wild type *74A* grown under glucose repression condition was used as common control for all microarray experiments. In order to assess dye bias effects, two sets of data were obtained in which cDNA from *74A* grown in glucose-rich medium were labeled with Cy3, whereas cDNA from *rco-3* or *dgr-1* were labeled with Cy5. One or two additional dye-swap experiments were performed in which the fluorescent dyes were switched during the labeling process. The tab-delimited data files from ScanAlyze were analyzed using GeneSpring (Silicon Genetics) for advanced analysis. First, the background value was subtracted from the raw data of each spot. Second, the subtracted expression data was filtered based on the value derived from the average value of the negative control spots to eliminate any spots that show signal intensity below the average value of negative control spots. The resulting data was normalized using the intensity dependent (Lowess) normalization

protocol, in which 20.0% of the data was used to calculate the Lowess fit at each point and the control value for each measurement was adjusted on the curve (GeneSpring Software, Silicon Genetics). Duplicate experiments were analyzed as individual experiments and reproducibility of replicate arrays was assessed using an experiment tree analysis in which the correlation coefficient of each gene across the set of experiments was quantitated to build the tree. The dye swap replicate experiments always branched together in the experiment tree indicating there is a subtle dye bias effect. Genes with two-fold or higher level changes in at least three of four or two of three duplicate experiments were flagged; this subset of genes was further filtered to identify genes that had a minimum two-fold differentiation in their averaged normalized value.

RESULTS

Phenotypic characterization of dgr-1 and rco-3 mutants

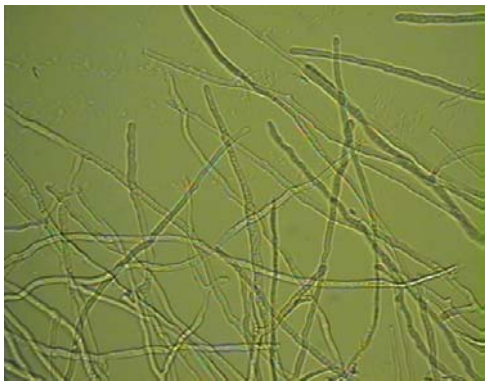
rco-3 is resistant to sorbose and 2-deoxyglucose (Madi *et al.,* 1997), in addition, *rco-3* gives rise to aerial hyphae on sorbose medium and allows production of mature conidia on sorbose medium. A previously isolated 2-deoxyglucose resistance mutant (*dgr-1*) (Allen *et al.,* 1989) is also resistant to sorbose and forms conidiophores on sorbose medium. When cultured in liquid medium with 2% glucose, wild type strains form filamentous hyphae while *rco-3* produces budding cells resembling conidiophores and free conidia can be observed (Figure 3.1). The *dgr-1* mutant displays a similar budding pattern but produces fewer conidiophores than *rco-3* (Figure 3.1). Hence, both

rco-3 and *dgr-1* mutants induce conidiation without carbon starvation. It is known that *rco-3* and *dgr-1* mutants posses reduced sugar transport activities relative to wild type (Allen *et al.,* 1989; Madi *et al.,* 1997; Chapter IV). Therefore, *rco-3* and *dgr-1* mutants provide a link between sugar metabolism and regulation of conidiation in *N. crassa*. The phenotypic resemblance prompted me to hypothesize that *rco-3* and *dgr-1* mutants are in the same regulatory pathway that regulates a common set of genes. In addition, genetic studies also support the hypothesis (Chapter IV) that *rco-3* and *dgr-1* may be members of a common regulatory circuit. To test this hypothesis, I examine the global gene expression patterns of *rco-3* and *dgr-1* strains in response to glucose status.

Global transcription response of rco-3 and dgr-1 in response to glucose status

A cDNA microarray consisting of 1364 unique genes was generated as previously described (Lewis *et al.,* 2002). mRNA from wild type cells starved for glucose for two hours and from *rco-3* and *dgr-1* mutants grown in the presence or absence of glucose were each assessed relative to wild type glucose grown cells. Three replicate array hybridizations including one dye-swap experiment were conducted for each test condition. The data are represented as the average ratio of individual test condition vs. glucose grown wild type mycelium. For example, *N. crassa* open reading frame (ORF) NCU06482.1 was represented by EST clone W17H2 and has most sequence similarity to Pda1 (pyruvate dehydrogenase) in *S. cerevisiae* (Table 2.1). The expression of NCU06482.1 was reduced approximately five folds when wild type was starved for glucose (74AG- 0.21), while in the *rco-3* mutants, NCU06482.1 displays a

74A



rco-3

dgr-1

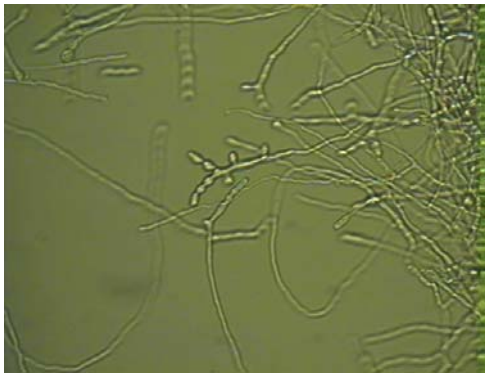


Figure 3.1 Conidiation of wild type, *rco-3* and *dgr-1* in liquid medium. Wild type (*74A*), *rco-3* and *dgr-1* strains were cultured in minimum medium with 2% glucose. *74A* grows primarily as filamentous hyphae while *rco-3* and *dgr-1* form conidial chains in submerged culture.

Table 3.1 *N. crassa* genes involved in central metabolism.

Ncr ORF [a]	EST ID [b]	Blast Match [c]	AG- [d]	RG+ [e]	RG- [f]	DG+ [g]	DG- [h]	Sce Homolog [i]
Glycolysis/Gluconeogenesis								
NCU06482.1	W17H2	Pyruvate dehydrogenase	0.21	0.64	0.25	0.65	0.3	Pda1
NCU03004.1	SC5E2	Pyruvate dehydrogenase	0.21	0.32	0.21	0.46	0.16	Pdb1
NCU02407.1	NM6A1	Dihydrolipoamide dehydrogenase	0.6	0.72	0.54	0.77	0.52	Lpd1
NCU06075.1	SM2G11	Pyruvate kinase	0.17	0.38	0.26	0.47	0.34	Pyk1
NCU06075.1	SC2A6	Pyruvate kinase	0.16	0.48	0.27	0.58	0.38	Pyk1
NCU06075.1	NC4C8	Pyruvate kinase	0.18	0.3	0.23	0.45	0.33	Pyk1
NCU10042.1	SC1H2	Enolase	0.35	0.75	0.75	0.64	0.51	Eno2
NCU10042.1	SP6F12	Enolase	0.4	0.54	0.61	0.6	0.53	Eno2
NCU02252.1	NC2D12	Phosphoglycero mutase	0.18	0.23	0.11	0.29	0.13	NH [j]
NCU02252.1	NC3B11	Phosphoglycero mutase	0.18	0.22	0.11	0.29	0.13	NH
NCU02252.1	SC2H9	Phosphoglycero mutase	0.16	0.29	0.1	0.3	0.13	NH
NCU07914.1	NC2D1	Phosphoglycerate kinase	1.36	0.42	0.86	0.4	0.71	Pgk1
NCU07914.1	NC3D6	Phosphoglycerate kinase	1	1.3	0.94	0.58	0.67	Pgk1
NCU01528.1	CCG-7	Glyceraldehyde 3-phosphate dehydrogenae	1.3	0.36	0.75	0.72	0.83	Tdh1
NCU01528.1	NC1G4	Glyceraldehyde 3-phosphate dehydrogenae	1.3	0.42	0.67	0.52	0.69	Tdh1
NCU01528.1	NC3C3	Glyceraldehyde 3-phosphate dehydrogenae	1	0.34	0.71	0.58	0.74	Tdh1
NCU01528.1	W08B11	Glyceraldehyde 3-phosphate dehydrogenae	1.2	0.38	0.72	0.67	0.79	Tdh1
NCU07550.1	SC1G8	Triosephosphate isomerase	0.43	0.23	0.53	0.27	0.57	Tpi1
NCU07550.1	NC2E4	Triosephosphate isomerase	0.44	0.24	0.53	0.29	0.55	Tpi1
NCU07807.1	SC3F5	Fructose-bisphosphate aldolase	0.19	0.93	0.17	0.76	0.17	Fba1
NCU02542.1	NC4B10	Hexokinase	0.49	0.55	0.64	0.6	1.1	Hxk2
NCU00575.1	W10H9	Glucokinase	0.54	1.1	0.53	1.5	0.79	Glk1
NCU09873.1	NM5C3	Phosphoenolpyruvate carboxykinase	33	4.3	6.4	6	5	Pck1
NCU09873.1	SM3H9	Phosphoenolpyruvate carboxykinase	29	3.5	4.4	3.7	3.7	Pck1
NCU09347.1	NP3H2	Fructose-2,6-bisphosphatase	1.7	0.57	0.63	0.63	0.63	Fbp26

Table 3.1 (continued)

Ncr ORF [a]	EST ID [b]	Blast Match [c]	AG-[d]	RG+[e]	RG-[f]	DG+[g]	DG-[h]	*Sce* Homolog[i]
Citrate Cycle (TCA Cycle)								
NCU01692.1	NM6F5	Citrate synthase	4.5	0.52	0.41	0.94	0.45	Cit1
NCU02366.1	SC3F7	Aconitate hydratase	3.75	0.92	0.74	1.1	0.88	Aco1
NCU00775.1	SC1C8	Isocitrate dehydrogenase	1.16	0.91	1.1	0.92	1.1	Idh1
NCU02438.1	NM1B4	Alpha-ketoglutarate dehydrogenase	0.8	0.89	0.85	0.74	0.56	Kgd2
NCU02407.1	NM6A1	Dihydrolipoamide dehydrogenase	0.6	0.72	0.54	0.77	0.52	Lpd1
NCU08471.1	W13H10	Succinyl-CoA ligase	0.3	0.45	0.32	0.62	0.44	Lsc 2
NCU08336.1	NC1F7	Succinate dehydrogenase	3.5	0.66	0.45	0.66	0.87	Sdh1
NCU00959.1	NM7H6	Succinate dehydrogenase	5.2	0.65	0.69	0.85	0.72	Sdh2
NCU03031.1	SC5F1	Succinate dehydrogenase	4.2	0.56	0.58	0.73	0.74	Sdh4
NCU10008.1	NM1B3	Fumarase	1.3	0.45	0.59	0.47	0.56	Fum1
NCU04899.1	W08E4	Malate dehydrogenase	4.5	0.89	0.78	1.1	0.83	Mdh1
NCU04899.1	W08B12	Malate dehydrogenase	3.1	0.86	0.67	1.2	0.77	Mdh1
Glyoxylate Cycle								
NCU02482.1	NP6C12	Citrate synthase	6.8	0.56	0.39	0.61	0.44	Cit2
NCU02366.1	SC3F7	Aconitate hydratase	3.75	0.92	0.74	1.1	0.88	Aco1
NCU02481.1	NM3F4	Isocitrate lyase	2.91	0.6	0.38	0.59	0.38	Icl1
NCU04230.1	NP3E9	Isocitrate lyase	10.8	1.1	1.1	1.2	1.1	Icl1
NCU03857.1	W17F2	NADP-dependent isocitrate dehydrogenase	2.77	0.57	0.33	0.83	0.47	Idp2
NCU06211.1	NP2E7	Malate dehydrogenase	5.98	0.93	4.4	0.91	4.1	Mdh2
Ethanol Metabolism								
NCU02193.1	NC3D2	Pyruvate decarboxylase	0.19	0.19	0.01	0.15	0.08	Pdc6
NCU02476.1	NP6D7	Alcohol dehydrogenase	1.1	1.4	1.5	1.5	1.1	Adh1
NCU01754.1	NC4C1	Alcohol dehydrogenase	3.79	0.37	0.82	0.43	1	Adh2
NCU01754.1	NC5E1	Alcohol dehydrogenase	3.88	0.47	0.72	0.32	0.83	Adh2
NCU09285.1	NC3D8	Alcohol dehydrogenase	1.5	1.7	3.6	1.1	2.2	Adh3

Table 3.1 (continued)

Ncr ORF [a]	EST ID [b]	Blast Match [c]	AG-[d]	RG+[e]	RG-[f]	DG+[g]	DG-[h]	*Sce* Homolog[i]
NCU09285.1	W01C6	Alcohol dehydrogenase	1.5	1.7	4.1	1.1	2.3	Adh3
NCU09266.1	SP3B11	Aldehyde dehydrogenase	7.45	0.83	1.46	0.78	1.57	Ald2
NCU08669.1	SC1C1	Aldehyde dehydrogenases	1.36	3.71	8.1	8.2	19	Ald3
NCU08669.1	SC1C5	Aldehyde dehydrogenase	1.4	1.2	2.3	1.5	1.8	Ald3
Pentose Phosphate Pathway								
NCU09111.1	NC3F8	Glucose-6-phosphate 1-dehydrogenase	0.38	0.88	0.72	0.81	0.73	Zwf1
NCU09111.1	W07G12	Glucose-6-phosphate 1-dehydrogenase	0.41	1.3	0.92	1.1	0.84	Zwf1
NCU09111.1	W09C4	Glucose-6-phosphate 1-dehydrogenase	0.35	1.4	0.67	0.94	0.74	Zwf1
NCU00519.1	W13F7	D-ribulose-5-Phosphate 3-epimerase	1.4	0.64	0.6	1.34	1	Rpe1
NCU01328.1	NC4B12	Transketolase	0.33	0.99	1.65	0.82	1.51	Tkl1
NCU02136.1	NC3G5	Transaldolase	0.27	0.34	1.15	0.69	0.94	Tal1
NCU02136.1	W10A6	Transaldolase	0.46	0.58	0.81	0.78	0.78	Tal1

[a] The *N. crassa* predicted ORFs are from the Whitehead Institute *Neurospora* sequence project
[b] The *N. crassa* ESTs are from *Neurospora* genome project at University of New Mexico
[c] Blast Match represent the most significant match in NCBI database (minimum e-value $1e^{-5}$)
[d] 74A G- is the average ratio of wild type glucose-deprived to wild type glucose-grown cells of replicate experiments.
[e] RG+ is the average ratio of the *rco-3* strain grown in the presence of glucose to wild type glucose-grown cells for replicate experiments.
[f] RG- is the average ratio of the *rco-3* strain grown in the absence of glucose to wild type glucose-grown cells for replicate experiments
[g] DG+ is the average ratio of the *dgr-1* strain grown in the presence of glucose to wild type glucose-grown cells of replicate experiments
[h] DG- is the average ratio of the *dgr-1* strain in the absence of glucose to wild type glucose-grown cells of replicate experiments
[i] *Sce* Homolog are the best hit using predicted *N. crassa* ORF to blast against NCBI *S. cerevisiae* database.
[j] NH indicates that the corresponding *N. crassa* ORF has no homologue in *S. cerevisiae*.

slightly declined expression in glucose rich medium compared to wild type glucose grown cells (RG+ 0.64) and glucose deprivation further repressed the transcription (RG- 0.25) (Table 3.1). The *dgr-1* mutants exhibit a similar regulation pattern for NCU06482.1 as *rco-3* strains (DG+ 0.65, DG- 0.3) (Table 3.1). Another example is *N. crass* ORF (NCU09873.1), which is represented by two EST clones (NM5C3 and SM3H9) (Table 3.1). NCU09873.1 is homologous to yeast Pck1 encoding phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis. NCU09873.1 was induced approximately 30-fold when the wild type strain was deprived glucose for two hours (74A G- 33, 28) (Table 3.1). *rco-3* and *dgr-1* strains display an elevated level of NCU09873.1 transcript in the glucose rich medium compared to wild type strain (RG+ 4.3, 3.5 and DG+ 6, 3.7) and glucose limitation can not further induce the elevated expression in *rco-3* and *dgr-1* strains (RG- 6.4, 4.4 and DG- 5, 3.7) (Table 3.1).

In general, *rco-3* and *dgr-1* display similar gene expression patterns for genes on the array. A total of 233 genes displayed elevated expression in *rco-3* or *dgr-1* strains compared to wild type mycelium grown in glucose-rich medium, whereas 269 unique genes displayed reduced transcript levels relative to wild type (Figure 3.2). Those ESTs with similarity to protein sequences in the NCBI non-redundant data base were assigned to functional groups according to MIPS functional categories (Figure 3.2). Comparing my list of approximately 500 regulated transcripts in *rco-3* and *dgr-1* to 210 genes differentially expressed in response to glucose in the wild type (Chapter II), it is clear that there is *rco-3*/*dgr-1* dependent regulation or novel glucose regulation that is observed in *rco-3*/ *dgr-1* backgrounds but not in wild type. In general, genes whose

transcript levels decreased in *rco-3* and *dgr-1* belonging to general biosynthetic pathways, including carbon metabolism (20%), amino acid biosynthesis (12%), lipid biosynthesis (9%) and protein biosynthesis (10%). In contrast, genes involved in degradation (18%) and conidiation (3%) are stimulated in *rco-3* and *dgr-1* mutants independent of glucose status. The expression patterns reflect the low level of sugar transport, the shift to conidiation and concomitant reduction in mycelium growth of *rco-3* and *dgr-1* mutants under test conditions.

Gene expression patterns in rco-3 and dgr-1: Central metabolism

Central metabolism is composed of glycolysis, the tricarboxylate cycle (TCA cycle), the glyoxylate cycle, and gluconeogenesis. The glycolytic cycle converts glucose into pyruvate and pyruvate can be channeled into fermentation to generate ethanol or completely degraded into carbon dioxide and water via the TCA cycle and the glyoxylate cycle. Previous study of glucose-regulated gene transcription profiles demonstrated that *N. crassa* catabolizes glucose simultaneously through respiration and fermentation in aerobic culture, a finding that is consistent with the measurements of ethanol production and enzyme activities in *N. crassa* (Chapter II). As mentioned above, *rco-3* and *dgr-1* have reduced glucose transport activities compared to wild type strain, therefore, comparing the central metabolism in *rco-3* and *dgr-1* mutants with wild type strain will help me to understand how glucose transport affects carbon catabolism in *N. crassa*.

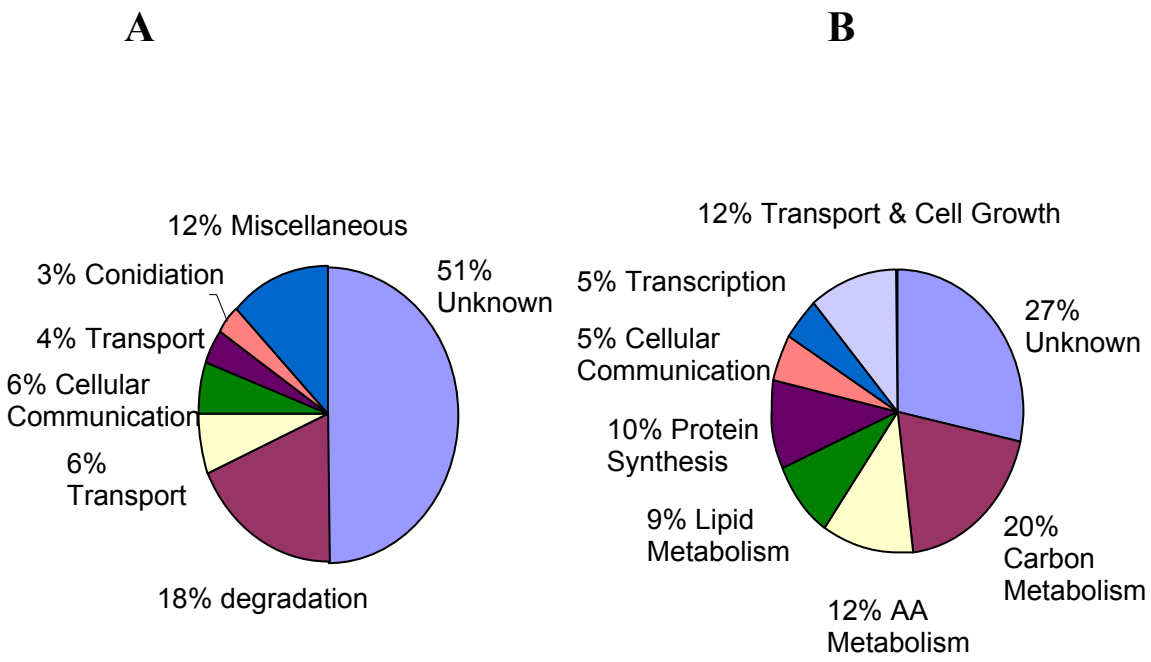


Figure 3.2 Summary of distribution of *N. crassa* genes differentially expressed in *rco-3* and *dgr-1* mutants. (A) Distribution of 233 unique transcripts induced in *rco-3* and/or *dgr-1* mutants. (B) Distribution of 269 unique genes repressed in *rco-3* and/or *dgr-1* mutants. Classification follows MIPS functional categories and has BLASTP hits (P<1e-5) to *S. cerevisiae* and the NCBI non-redundant database. The unknown category represents sequences with no significant similarity to proteins in the database. The miscellaneous category refers to genes involved in a variety of other processes.

Twenty four transcripts representing twelve genes in glycolysis are either down-regulated or unaffected in *rco-3* and *dgr-1* mutants in glucose rich medium compared to wild type strains (Table 3.1) (Figure 3.3). Previous analysis indicates that transcriptional regulation of genes in the central metabolism correlates well with the regulation of enzyme activities in *N. crassa* (Chapter II). Therefore the reduced transcript levels for glycolytic enzymes may reflect a decreased capacity of glucose utilization, which is consistent with the reduced glucose transport activities in *rco-3* and *dgr-1* mutants. Consistent with this view, glucose deprivation further represses the transcript levels of some glycolytic genes in *rco-3* and *dgr-1* mutants (Table 3.1). Pyruvate decarboxylase is a key enzyme that catalyzes the rate-limiting step of ethanol production. The transcript for pyruvate decarboxylase in *rco-3* and *dgr-1* mutants under glucose repression condition were expressed at the same low level as wild type grown in the absence of glucose and this expression was further reduced when *rco-3* and *dgr-1* were starved for glucose (Table 3.1). Although the gene encoding *N. crassa* alcohol dehydrogenase that most resembled to *S. cerevisiae* Adh1 (alcohol dehydrogenase I) was constitutively expressed in both *rco-3* and *dgr-1* mutants (Table 3.1) (Figure 3.3), the reduced level of pyruvate decarboxylase suggests that ethanol production in *rco-3* and *dgr-1* mutants is much lower than that in wild type. In contrast, genes encoding ethanol utilization enzymes including alcohol dehydrogenase (Adh2 and Adh3) and aldehyde dehydrogenase (Ald2 and Ald3) were expressed at relatively high levels in both mutants independent of glucose status (Table 3.1). However, potential alternative alcohol

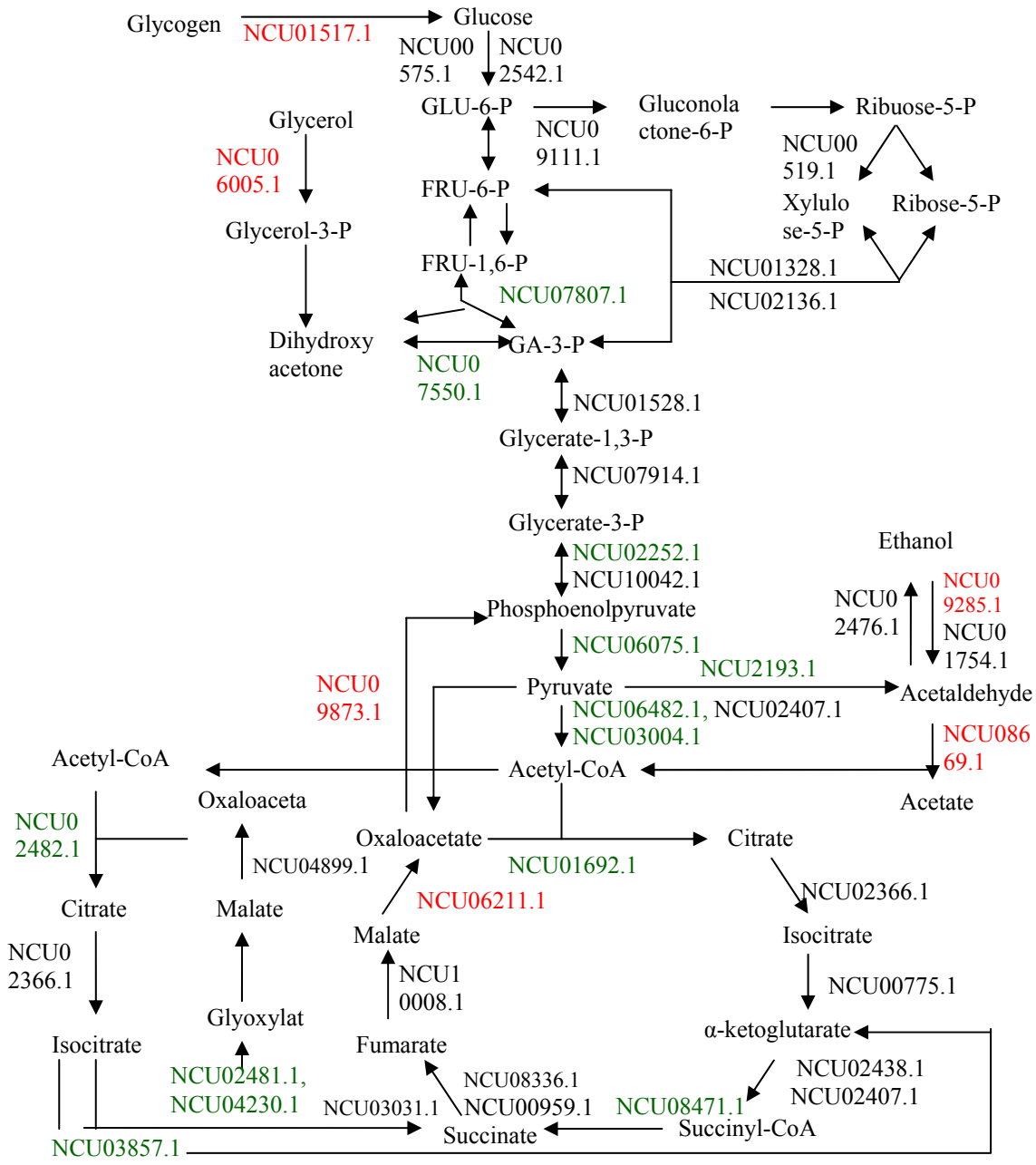


Figure 3.3 The *N. crassa* genes involved in central metabolism pathways. Only the key metabolic intermediates are indicated. Double-headed arrow denotes reversible reaction. The *N. crassa* genes encoding the enzyme that catalyze each step are indicated by the predicated ORF. Red letters indicate genes whose expression is induced in *rco-3* and *dgr-1* mutants compared to wild type glucose-grown cells. Green letters represents genes that are repressed in *rco-3* and *dgr-1* mutants relative to wild type glucose-grown cells. Black letters stands for genes unaffected by *rco-3* and *dgr-1* in the experiments.

dehydrogenase and aldehyde dehydrogenase genes may be expressed in *rco-3* and *dgr-1* to provide alternative enzymes to carry out these steps (Table 3.1). For example, NCU09266.1 and NCU08669.1 are two genes homologous to yeast aldehyde dehydrogenase. The transcription of NCU09266.1 was elevated seven fold when wild type cells were starved for glucose but was unaffected in *rco-3* and *dgr-1* mutants (Table 3.1). On the other hand, *rco-3* and *dgr-1* significantly increase the expression of NCU08669.1 and glucose deprivation further stimulates its expression (Table 3.1). But NCU08669.1 transcription was constitutively expressed in wild type regardless of glucose status (Table 3.1). Therefore, different isoforms of alcohol dehydrogenase and aldehyde dehydrogenase may play major roles in wild type than *rco-3* and *dgr-1* mutants. However, I notice that two ESTs representing NUC08669.1 show different expression patterns in microarray analysis, which could be arisen from several reasons as I discuss in Chapter V.

The pyruvate dehydrogenase enzyme complex catalyzes the rate limiting step to support respiration (Figure 3.3). The reduced expression of genes involved in the pyruvate dehydrogenase complexes in *rco-3* and *dgr-1* indicate less flow of acetyl-CoA into the TCA cycle even for cells grown in glucose rich medium (Table 3.1). Consistent with this view, sixteen genes involved in the TCA cycle and the glyoxylate cycle are either down-regulated or unaffected in *rco-3* and *dgr-1* mutants regardless of glucose level relative to glucose grown wild type cell (Table 3.1). The only exception is malate dehydrogenase II, which displays the same expression pattern in *rco-3* and *dgr-1* mutants as in the wild type strain (Table 3.1), which indicates that glucose regulation of

malate dehydrogenase II transcript level is independent of *rco-3* and *dgr-1*. The expression patterns of genes involved in the TCA cycle and the glyoxylate cycle indicate a reduced respiration rate in *rco-3* and *dgr-1* mutants.

Elevated transcription of genes in the TCA cycle and the glyoxylate cycle is indicative of activation of gluconeogenesis (Chapter II). Here, the low level expression of genes for citrate synthase, aconitate hydratase, isocitrate lyase, NADP-isocitrate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase I suggest that the gluconeogenesis pathway was not activated in *rco-3* and *dgr-1* mutants, even in the absence of glucose. However, one of the key enzymes in gluconeogenesis, phosphoenolpyruvate carboxykinase (NCU09873.1), was increased four-fold in *rco-3* and *dgr-1* relative to glucose-grown wild type cell and the expression were supported by Northern blot analysis (Table 3.1) (Figure 3.4). However, another key enzyme of gluconeogenesis, fructose 2,6 bisphosphatase, which is slightly induced when wild type cells are starved for glucose, displays reduced expression in *rco-3* and *dgr-1* mutants (Table 3.2). Taken together, *rco-3* and *dgr-1* may have a constitutively partially derepressed gluconeogenesis that is uninducible by glucose deprivation. If *rco-3* and *dgr-1* simply has lower sugar transport rate, I would expect that regulation of the gluconeogenesis upon glucose starvation should be the same as wild type.

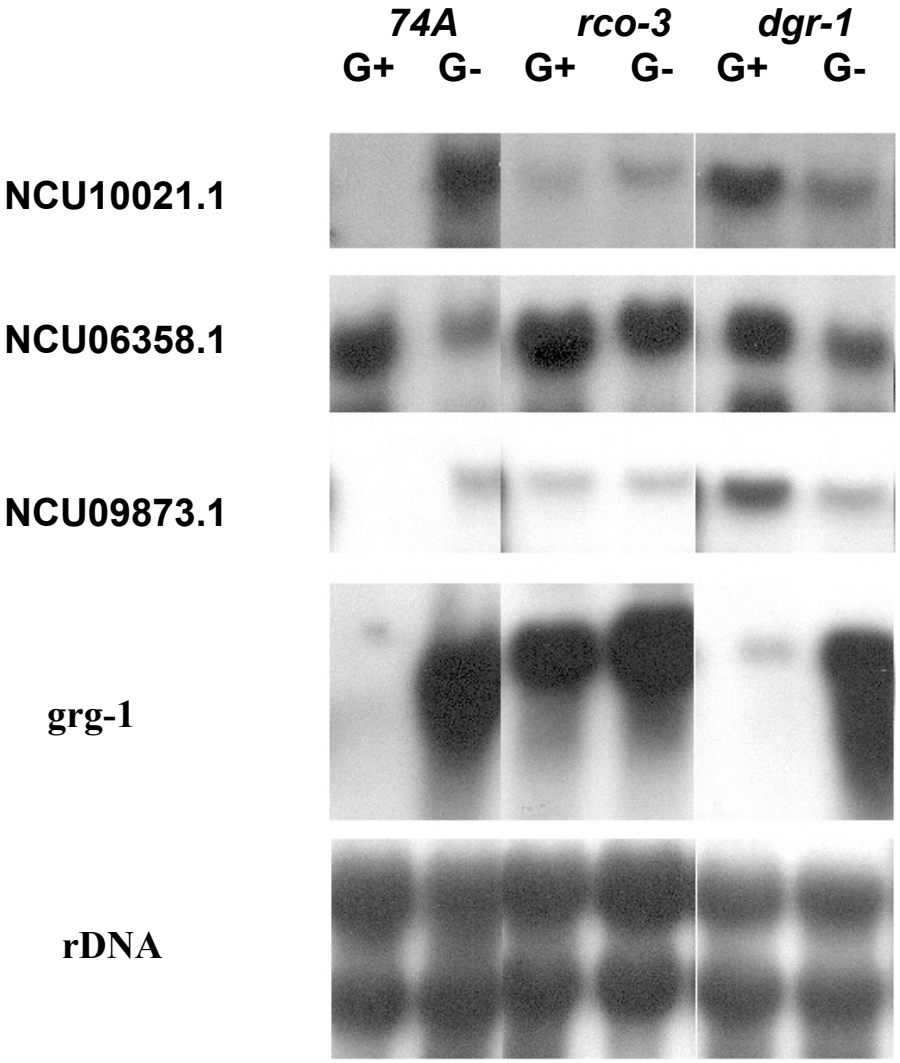


Figure 3.4 Several genes are differentially regulated by glucose, peptone, *rco-3* and *dgr-1*. Wild type (*74A*), *rco-3* and *dgr-1* strains were grown in liquid minimal medium supplemented with 2% peptone and 2% glucose for approximately 18 hours, mycelium were harvested by filtration and inoculated into minimal medium containing 2% glucose (G+) or medium lacking glucose (G-) for two hours. Genes that are not identified in *N. crassa* are indicated by predicted open reading frame. Known genes in *N. crassa* are represented by the gene name.

Lipid and amino acid metabolism

In *N. crassa* fatty acid beta oxidation and the glyoxylate cycle are co-localized in the glyoxysome. During glucose deprivation, fatty acid degradation and the glyoxylate cycle genes were coordinately activated (Chapter II). Several genes encode enzymes involved in fatty acid degradation, such as hydratase-dehydrogenase, 3-ketoacyl-CoA thiolase, 2, 4-dienoyl-CoA reductase and long-chain fatty acid-CoA ligase display increased expression when wild type cells are starved for glucose (Table 3.2), however, the transcription of these genes is either unaffected or only slightly induced in *rco-3* and *dgr-1* mutants regardless of glucose status (Table 3.2). As I discussed earlier, genes involved in the glyoxylate cycle and the TCA cycle were expressed at low level even when *rco-3* and *dgr-1* cells were starved for glucose. Therefore the behaviors of genes involved in lipid degradation and the glyoxylate cycle in *rco-3* and *dgr-1* mutants further confirm the coregulation between these two metabolic pathways.

In contrast to the unaffected activity of lipid degradation, the expression of genes in lipid biosynthesis was dramatically repressed in the *rco-3* and *dgr-1* strains. For example, several genes homologous to yeast ergosterol biosynthetic genes (*erg9*, *erg10*, *erg6*, *erg25*) had reduced expression in *rco-3* and *dgr-1* (Table 3.2), indicating a reduction in ergosterol biosynthesis. Yeast *ole1* gene encodes delta-9-fatty acid desaturase, a key enzyme for unsaturated fatty acid biosynthesis in *S. cerevisiae*. The transcript of *ole1* homolog in *N. crassa* was reduced ten fold compared to wild type glucose-grown cells when *rco-3* and *dgr-1* were starved for glucose (Table 3.2), suggesting a strong inhibition of fatty acid biosynthesis in *rco-3* and *dgr-1* mutants.

Table 3.2 *N. crassa* genes involved in lipid metabolism.

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	Sce Homolog
Fatty Acid Synthesis								
NCU08976.1	W07H5	Polyunsaturated fatty acid elongation enzyme	0.82	0.22	0.08	0.22	0.07	Fen1
NCU03492.1	NP6F6	Fatty acid hydroxylase	0.42	0.47	0.23	0.81	0.26	Scs7
NCU05259.1	NC3E10	Delta-9-fatty acid desaturase	0.19	0.48	0.21	0.76	0.17	Ole1
NCU05259.1	SC3E2	Delta-9-fatty acid desaturase	0.19	0.23	0.09	0.58	0.07	Ole1
NCU05259.1	SM4H12	Delta-9-fatty acid desaturase	0.19	0.26	0.07	0.72	0.07	Ole1
NCU08685.1	SC2A3	Phosphoribosylformylglycinamidine synthase	0.81	0.29	0.15	0.29	0.13	Ade6
NCU06054.1	SC3A10	Squalene synthase	0.91	0.55	0.54	0.98	0.52	Erg9
NCU02571.1	NC4D1	Acetyl-CoA C-acetyltransferase	0.26	0.41	0.37	0.45	0.33	Erg10
NCU03006.1	NM5B12	Sterol c-methyltransferase	0.25	0.53	0.26	0.71	0.19	Erg6
NCU06402.1	SC2F1	C-4 sterol methyl oxidase	0.3	0.4	0.21	0.39	0.19	Erg25
NCU05008.1	W13E4	NADH dehydrogenase	1.38	0.6	0.35	0.76	0.44	Acp1
NCU07719.1	SP7C9	Isopentenyl-diphosphate delta-isomerase	0.69	0.45	0.34	0.65	0.34	Idi1
NCU07859.1	SM2D12	Cyclopropane-fatty-acyl-phospholipid synthease	0.45	0.61	0.44	0.84	0.68	NH [b]
NCU04610.1	SP4C7	Oxysterol binding protein	1.1	2.1	1.7	1.4	1.5	Osh3
Fatty Acid Degradation								
NCU08828.1	NM4H8	Peroxisomal hydratase-dehydrogenase	5.13	0.9	1.96	1.22	1.46	Fox2
NCU08828.1	NP2E8	Peroxisomal hydratase-dehydrogenase	5.5	1.1	2.4	1.3	1.8	Fox2
NCU04796.1	NM5E11	Peroxisomal 3-ketoacyl-CoA thiolase	4.53	1.68	4	1.84	4.8	Fox3
NCU03893.1	SP4C8	2,4-dienoyl-CoA reductase	2.36	1.28	0.77	1.57	1.73	Sps19
NCU04380.1	SP1D3	Long-chain fatty acid--CoA ligase	1.2	1.37	0.93	1.6	0.86	Faa1
NCU01654.1	NM7A4	Long-chain fatty acid--CoA ligase	2.7	1.2	1.8	1	1.6	Faa2
NCU03929.1	SP6C11	Long-chain fatty acid--CoA ligase	1	0.86	1.2	1.1	0.99	Faa4
NCU00580.1	SC5C9	Acyl-CoA thioesterase	0.98	0.58	0.61	0.46	0.56	Tes1
NCU00580.1	NC2F3	Acyl-CoA thioesterase	1.11	0.62	0.44	0.48	0.44	Tes1
NCU00580.1	NM4B3	Acyl-CoA thioesterase	5.1	0.74	1.3	0.79	0.81	Tes1

Table 3.2 (continued)

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	*Sce* Homolog
NCU02960.1	NP5F8	Peroximal targeting signal receptor	3.03	2.08	2.83	1.73	2.37	Pas10
NCU07915.1	SM1D5	Peroxisomal organization and biogenesis protein	6.71	3.93	15.8	3.48	10.4	Yor292c
NCU00173.1	SP4E11	Esterase	1.82	0.49	0.75	0.4	0.7	Yjl068c
NCU03151.1	SC5D10	Peroxisomal membrane protein	0.3	0.48	0.38	0.58	0.3	Ahp1
NCU05006.1	NP3G10	Fatty acid omega-hydroxylase	1.43	4.15	3.6	1.76	3.31	NH
NCU00316.1	NM7A7	Adenine nucleotide transporter	4.7	0.87	1.2	1.2	1.5	Ant1
Lipid Transport								
NCU02263.1	W17F1	Sec14	0.5	0.78	0.52	0.87	0.49	Sec14
NCU03372.1	SP3B10	Sterol carrier protein	6.5	0.82	1.4	0.86	1.8	NH

[a] The columns in this table are the same as Table 3.1.
[b] NH indicate that corresponding *N. crassa* ORF has no homologue in *S. cerevisiae*.

Several amino acid biosynthetic pathways represented by multiple genes on the microarray were coordinately down-regulated in *rco-3* and *dgr-1* mutants (Table 3.3). Previous studies indicate that methionine, cysteine, valine, leucine and isoleucine biosynthetic pathways are expressed at low level when *N. crassa* cell were starved for glucose for two hours (Chapter II). These amino acid pathways are also suppressed in *rco-3* and *dgr-1* mutants even under glucose repression conditions (Table 3.3). In addition, genes that encode enzymes participating in arginine, histidine and glutamine biosynthesis display decreased transcript level in the *rco-3* and *dgr-1* mutants (Table 3.3). Overall, both lipid and amino acid biosynthesis appear to be reduced in *rco-3* and *dgr-1* mutants.

Conidiation and blue light regulation

Several conidiation specific genes (*con* genes, *fluffy* and *eas*) have been identified in *N. crassa* and their expression patterns during conidiation have been examined (Ebbole, 1996). *rco-3* and *dgr-1* induce conidiation without nutrient limitation in submerged culture. Therefore, it is expected that conidiation-specific genes should display increased transcript levels in *rco-3* and *dgr-1* mutants. The transcription of *con-6*, *con-10*, *fluffy* and *eas* are unaffected or slightly induced when wild type cells are starved for glucose for two hours (Table 3.4). However, in *rco-3* and *dgr-1* mutants, these genes have significantly elevated expression even under glucose repression and glucose deprivation further induced their transcript levels (Table 3.4).

Table 3.3 *N. crassa* genes involved in amino acid metabolism.

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	Sce Homolog
Met, Cys Biosynthesis								
NCU01882.1	NP4D9	Sulfate permease	0.47	0.65	0.37	0.95	0.43	NH [b]
NCU01985.1	SP1D2	ATP sulfurylase	0.25	0.83	0.46	1.17	0.9	Met3
NCU02005.1	SC5E11	phosphoadenosine phosphosulfate reductase	4	0.86	1.02	1.05	1.11	Met16
NCU02657.1	W13G12	Methionine adenosyltransferase	0.28	0.45	0.11	1.02	0.15	Sam2
NCU04077.1	W01C7	Sulfite reductase	0.31	0.61	0.2	0.77	0.23	Met10
NCU06512.1	NC1D10	Methionine synthase	0.96	0.57	0.56	0.93	0.49	Met6
NCU07930.1	W07A2	Adenosyl homocysteinase	0.73	0.49	0.35	0.93	0.58	Sah1
NCU07930.1	SC2C3	Adenosyl homocysteinase	0.64	0.22	0.29	0.72	0.43	Sah1
NCU09230.1	NC3A10	Cystathionine gamma-lyase	0.87	0.79	0.34	1.08	0.35	Str1
NCU09896.1	NC4E1	Adenylylsulfate kinase	0.74	0.42	0.36	0.51	0.4	Met14
Val, Leu, Ile Biosynthesis								
NCU01666.1	NC5G2	Acetolactate synthase	0.3	0.56	0.25	0.67	0.22	Ilv6
NCU01666.1	SM2F8	Acetolactate synthase	0.5	0.41	0.19	0.47	0.21	Ilv6
NCU03608.1	SC1H9	Ketol-acid reductoisomerase	0.92	0.33	0.12	0.28	0.1	Ilv5
NCU03608.1	SC2F2	Ketol-acid reductoisomerase	0.15	0.47	0.15	0.73	0.11	Ilv5
NCU06232.1	NP2D4	Isopropylmalate dehydrogenase	0.21	0.47	0.43	0.55	0.39	Leu2
NCU03575.1	NC1F12	isoleucyl-tRNA synthetase	0.41	0.73	0.61	0.86	0.66	Ils1
Arginine biosynthesis								
NCU00567.1	NC1G8	Arg-6 protein	0.84	0.76	0.39	0.39	0.22	Arg6
NCU08162.1	W17B9	Argininosuccinate lyase	0.22	0.73	0.4	1.12	0.39	Arg4
NCU02639.1	NC3G2	Argininosuccinate synthase	0.18	0.49	0.32	0.92	0.4	Arg1
NCU07732.1	NC2C3	carbamoyl-phosphate synthase	0.54	0.71	0.42	1.19	0.37	Cpa1

Table 3.3 (continued)

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	Sce Homolog
Glutamine Biosynthesis								
NCU01195.1	NM2B7	NADP-dependent glutamate dehydrogenase	0.76	0.86	1	0.54	1.4	Gdh1
NCU06724.1	NC5G11	Glutamine synthetase (nitrogen fixation)	0.79	0.7	0.22	0.98	0.31	Gln1
NCU06724.1	NC4F5	Glutamine synthetase (nitrogen fixation)	0.79	0.7	0.22	0.98	0.26	Gln1
NCU03076.1	SC7G11	Delta-1-pyrroline-5-carboxylate dehydrogenase	0.87	0.38	0.23	0.23	0.27	Put2
NCU04720.1	W17B4	Nitrite reductase (NADPH)	1.1	0.82	0.93	0.48	0.74	NH
NCU04720.1	W17D11	Nitrite reductase (NADPH)	0.63	0.74	0.41	0.41	0.31	NH
NCU00461.1	W01E10	NAD-dependent glutamate dehydrogenase	9.55	4.55	5.66	2.62	6.18	Gdh2
Histidine Biosynthesis								
NCU03139.1	W08H2	Histidine biosynthesis trifunctional protein	0.79	0.28	0.12	0.27	0.1	His4
NCU09320.1	W01H2	ATP phosphoribosyltransferase	0.57	0.51	0.42	0.71	0.55	His1
Miscellaneous AA Biosynthesis								
NCU02274.1	NC4E5	Serine hydroxymethyltransferase	0.97	0.57	0.47	0.97	0.52	Shm2
NCU02274.1	SC6B7	Serine hydroxymethyltransferase	0.44	0.55	0.47	1.54	0.63	Shm2
NCU01830.1	NP3D4	4-hydroxyphenylpyruvate dioxygenase	5	1.02	2.52	1.01	4.48	NH
NCU03118.1	W17D3	saccharopine dehydrogenase	0.65	0.58	0.3	0.7	0.41	Lys1
NCU04411.1	NC4D5	Phosphoribosylanthranilate transferase	0.86	0.59	0.69	0.8	0.66	Trp4
NCU05420.1	SC5B3	Chorismate synthase/flavin reductase	0.44	0.62	0.5	1.05	0.36	Aro2
NCU03935.1	SC2C6	Homoserine dehydrogenase	0.45	0.84	0.6	0.69	0.53	Hom6
NCU01821.1	NP4E5	Similarity to transaminases	1.67	0.48	0.56	0.54	0.61	Yfl030w
NCU07266.1	NM6G8	Diphthamide synthase	0.61	1.2	2.2	0.98	1.9	Dph5
NCU08287.1	W17A5	Carbamoyl-phosphate synthase	0.7	0.62	0.39	1.14	0.52	Ura2
NCU06017.1	SM1A12	mercaptopyruvate sulfurtransferase	5.4	6.25	6.99	2.29	3.11	Yor251c
NCU07176.1	NM8H12	L-arginine:lysine amidinotransferase	1.31	2.68	3.81	1.5	3.81	NH

Table 3.3 (continued)

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	Sce Homolog
AA degradation								
NCU03648.1	SP6H12	glutaminase A	1.64	0.5	1.03	1.11	1.16	NH
NCU04133.1	NM6B10	Aminopeptidase Y homolog	1.14	1.89	2.55	1.73	2.19	Ybr074w
NCU01757.1	NM5A10	Asparaginase related protein	8.96	6.03	11.43	2.06	4.96	NH
NCU05387.1	SP4G9	Nitrilase	1.6	0.56	1	0.47	1.2	Nit1
NCU08877.1	SM4H11	Glycine cleavage system H protein	2.93	0.49	0.38	0.75	0.46	Gcv3
AA Transport								
NCU08066.1	NM1C12	amino acid transport proteins	0.87	3.2	7.7	3.3	4.9	Yer119c
NCU01810.1	NC4D3	amino acid transporter, mitochondrial carrier protein	0.63	0.59	0.22	0.58	0.26	Ort1

[a] The columns in this table are the same as Table 3.1.
[b] NH indicates that the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

Table 3.4 *N. crassa* genes involved in conidiation and light induction.

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	Sce Homolog
NCU08769.1	CON-6	Conidiation specific protein-6	3.5	4.5	27	1.4	14	NH [b]
NCU08769.1	pCON 6	Conidiation specific protein-6	1.3	2.3	6.8	1.9	4.6	NH
NCU07325.1	CON-10	Conidiation specific protein-10	2.3	7.3	12	2.4	3.2	NH
NCU07325.1	pCON 10	Conidiation specific protein-10	1.6	5.5	11	2.8	3.4	NH
NCU08726.1	FL	Conidial development protein fluffy	0.81	1.5	2.2	2.1	2.1	NH
NCU08726.1	FL 1/2	Conidial development protein fluffy	0.77	1.7	2.1	2	2	NH
NCU08726.1	FL 1/4	Conidial development protein fluffy	0.61	0.98	1.3	2.3	2.2	NH
NCU08457.1	CCG-2	Hydrophobin	1.52	12	17	6.9	11	NH
NCU08457.1	CCG-2 1/2	Hydrophobin	0.75	17	31	9	16	NH
NCU08457.1	CCG-2 1/4	Hydrophobin	0.77	16	24	8.5	18	NH
NCU08457.1	NC5E3	Hydrophobin	0.79	20	15	6.6	12	NH
NCU08457.1	SC2A2	Hydrophobin	0.8	12	18	5.8	11	NH
NCU08936.1	NM5F5	Sporulation-specific protein	1.1	2.9	2.6	2.1	1.8	Sps2
NCU08936.1	NP3E12	Sporulation-specific protein	1.2	1.6	2.8	1.8	1.9	Sps2
NCU08936.1	NP3H6	Sporulation-specific protein	1.2	2.2	2.7	1.8	2	Sps2
NCU08936.1	NP4F8	Sporulation-specific protein	1	1.8	2.7	2.1	1.5	Sps2
NCU08936.1	NP5C3	Sporulation-specific protein	1.1	2	3.1	2	1.5	Sps2
NCU09057.1	SP4D11	Embryo-specific protein	3.9	3.6	7.5	2.5	3.6	No
NCU03753.1	CCG-1	Glucose-repressible protein (grg-1)	3.6	5.3	6.6	2.3	3.7	NH
NCU03753.1	NM6B6	Glucose-repressible protein (grg-1)	8.8	13	21	4.4	9.1	NH
NCU03753.1	NP5G7	Glucose-repressible protein (grg-1)	14	19	29	5.8	19	NH
NCU03753.1	SP6C9	Glucose-repressible protein (grg-1)	10	14	26	4.8	11	NH
NCU07267.1	SM4C4	BLI-3 protein (stress protein)	2.7	2.6	13	1	7.6	NH
NCU10055.1	NM4H11	Opsin-1 (nop-1)	2.5	3.6	14	1.8	6.8	Mrh1
NCU02356.1	WC-1	White collar 1 (wc-1)	0.97	0.62	0.37	0.53	0.44	Gat2
NCU03967.1	W07H6	VVD	2.83	1.1	1.8	1.6	2.7	NH

[a] The columns in this table are the same as Table 3.1.
[b] NH indicates that the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

Several other genes also display a similar expression pattern as conidiation specific genes (Table 3.4), including *grg-1*, a glucose repressible gene with unknown function, *bli-3*, a blue light induced gene with unknown function. The elevated regulations of *grg-1* by *rco-3* and *dgr-1* mutants were confirmed by Northern hybridization (Figure 3.4). Previous studies demonstrate that *con-6*, *con-10* and *bli-3* are rapidly induced by blue light and *grg-1* and *eas* shows a late induction after exposure to light (Linden *et al.,* 1997). In addition, *rco-3* and *dgr-1* have altered expression of the genes encoding opsin (*nop-1*) (Bieszke *et al.,* 1999), *wc-1* (white collar 1, a blue light sensor) (Froehlich *et al.,* 2002**)** and *vivid* (a gene regulating light responses) (Heintzen *et al.,* 2000) (Table 3.4). Taken together, *rco-3* and *dgr-1* may be involved in the light regulation machinery in *N. crassa*.

Transport

rco-3 and *dgr-1* have altered sugar transport activities compared to wild type strain, which is likely due to regulation of glucose transporter genes rather than *rco-3* or *dgr-1* directly acting as sugar transporters Four predicted hexose transporter homologs were present on the array and their transcript levels were differentially regulated in *rco-3* and *dgr-1* mutants (Table 3.5). Two of them, NCU10021.1 and NCU08114.1 are highly induced when wild type cells are starved for glucose but have a high basal transcript level that is un-inducible in the *rco-3* and *dgr-1* mutants (Table 3.5). The expression pattern of NCU10021.1 was verified by Northern blot analysis (Figure 3.4) and is similar to the measurement of high affinity glucose transport activity in *rco-3* and *dgr-1*

Table 3.5 *N. crassa* genes involved in transport.

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	Sce Homolog
NCU00450.1	NP5B3	Sucrose carrier	1.2	0.63	1.9	1.2	2.6	
NCU00988.1	NM5H7	Hexose transporter	4	3.5	4.9	2.4	4.3	
NCU06358.1	NP4E6	Hexose transporter	0.89	3.7	6.7	3.6	5.7	
NCU10021.1	SP1A4	Hexose transporter	10.6	1.2	1.7	2.9	2.5	
NCU08114.1	SM4A3	Hexose transporter	7.5	1.8	1.7	1.2	1.1	
NCU05089.1	NP3E1	Monocarboxylate permease	2.6	0.97	3.4	1.2	3.1	Mch5
NCU09698.1	SP4D10	MFS allantoate permease, nicotinic acid	1.9	3.1	11	2.7	18	Tna1
NCU09698.1	W17H1	MFS allantoate permease	1.7	6.1	9.9	2.7	11	Tna1
NCU00199.1	NC4A6	nucleoporin (nuclear transport)	1	0.36	0.76	0.5	0.71	Nup188
NCU04274.1	SC3F4	Sts1- transport (RNA transport)	2.5	1.1	1.8	1.1	1.8	Sts1
NCU05226.1	NM5E9	membrane protein (ABC transport)	0.53	0.95	0.51	0.76	0.41	Ydr061w
NCU06702.1	NM1C1	vesicular transport	0.36	0.9	0.99	0.87	1.4	Yip2
NCU07319.1	SC1F12	coatomer beta chain of secretory pathway	0.47	0.8	0.55	0.89	0.61	Sec27
NCU01470.1	NM5H5	putative vacuolar protein	7	4.9	7	1.8	3.1	Vac8
NCU00790.1	W01B10	potassium transporter	1.1	0.51	0.33	0.9	0.48	NH
NCU08055.1	NP3E3	high-affinity zinc transport	1	4.3	2.5	2.5	1.3	NH
NCU06380.1	W06E6	membrane protein (Zinc transporter)	1	2.2	1.5	1.2	1.2	Yil023c
NCU03368.1	SM4C9	antioxidant & metal homeostasis	0.48	1	1	0.63	0.84	Atx1
NCU01207.1	NM7G11	Vacuolar ATP synthase (iron transport)	2.1	2.6	3.7	1.4	2.4	Tfp1
NCU01680.1	SP6E7	H+-transporting ATPase	0.64	1.1	0.53	2.1	0.39	Pma1
NCU02887.1	NP2B6	putative K+ channel	1.3	0.99	2	0.69	1.6	Ypl088w
NCU00430.1	NP3H3	Na+/H+ exchanging protein	0.78	2.1	0.72	2.2	0.76	Nha1

[a] The columns in this table are the same as Table 3.1.
[b] NH indicates that the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

mutants. NCU10021.1 has been demonstrated to encode a high-affinity glucose transporter in *N. crassa* (Chapter IV). Expression of another glucose transporter homolog, NCU06358.1, is weakly decreased in wild type cell starved for glucose but was constitutively expressed in *rco-3* and *dgr-1* strains in Northern blot analysis (Figure 3.4). However, microarray data indicate that the expression of NCU06358.1 is induced in *rco-3* and *dgr-1* independent of glucose compared to wild type glucose-grown cell (Table 3.5). There are discrepancy between microarry data and Northern blot analysis, which can be due to several reasons as I discuss in Chapter V. NCU00988.1 encodes another glucose transporter homolog that displays elevated expression under glucose starvation and in the *rco-3* and *dgr-1* mutant backgrounds (Table 3.5). One interesting observation is that a transcript homologous to yeast *Tna1* shows marginally increased expression in wild type cells deprived for glucose but displays dramatically elevated transcript level in *rco-3* and *dgr-1* mutants (Table 3.5). *Tna1* encode a high affinity nicotinic acid permease (Llorente and Dujon, 2000). The activation of *Tna1* homolog might reflect an increased ability to take up nicotinic acid in *rco-3* and *dgr-1* mutants.

Cell growth

rco-3 and *dgr-1* have reduced biomass accumulation compared to wild type cells under test conditions, presumably due to the developmental induction of conidiation. Consistent with this observation, a set of genes involved in cell growth are down-regulated in *rco-3* and *dgr-1* mutants. Numerous genes encoding translation initiation factor, elongation factors, ribosomal protein, cytoskeleton components as well as vitamin

Table 3.6 *N. crassa* genes involved in general biosynthesis.

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	*Sce* Homolog
Protein Synthesis								
NCU00464.1	W08D10	60s ribosomal protein L32	0.67	0.63	0.48	1	0.78	Rpl32
NCU00235.1	SM4F10	ATP-dependent Helicase	2.7	0.5	0.5	0.74	0.5	Drs1
NCU00258.1	SC5A9	40S ribosomal protein	0.42	0.85	1.1	0.78	1.6	Rps7a
NCU02208.1	SP6G12	translation initiation factor eIF3	0.43	1	0.49	1.3	0.5	PRT1
NCU02905.1	W09C7	ribosomal protein L23	0.76	0.59	0.42	0.85	0.76	Rpl23a
NCU03826.1	NC1A6	elongation factor 1 (EF-1)	0.4	0.65	0.85	0.83	1	Cam1
NCU03826.1	NM6D9	elongation growth 1-gamma	0.25	0.9	0.76	1.1	1.2	Cam1
NCU04806.1	NP3E11	30s ribosomal protein	0.8	0.62	0.4	0.44	0.46	Rsm7
NCU05599.1	SM1F12	40s ribosomal protein 28s	0.77	0.69	0.5	1.1	0.84	Rps28b
NCU05599.1	W09H4	40s ribosomal protein 28s	0.74	0.64	0.45	0.85	0.78	Rps28b
NCU06035.1	NC1A10	translation elongation factor eEF-1	0.43	0.58	1.3	1	1.2	Efb1
NCU06226.1	NC2D3	ribosomal protein L23	0.87	0.54	0.38	0.96	0.72	Rpl25
NCU06226.1	NC3G1	ribosomal protein L23	0.72	0.53	0.35	0.92	0.77	Rpl25
NCU06226.1	SC2F4	ribosomal protein L23	0.81	0.63	0.39	1	0.84	Rpl25
NCU06431.1	NM8G12	40S ribosomal protein S22	1.1	0.83	0.46	1.2	0.82	Rps22a
NCU06432.1	W15D5	40S ribosomal protein S12	1.5	1.1	1.3	1.6	2.1	Rps12
NCU06469.1	NM5F4	60s ribosomal protein	0.75	0.62	0.46	0.87	0.5	Ygl068w
NCU06661.1	NC3E8	60s ribosomal protein l22	0.63	0.74	0.43	0.91	0.76	Rpl22a
NCU06661.1	NM2A10	60s ribosomal protein l22	0.72	0.6	0.4	0.98	0.82	Rpl22a
NCU06743.1	NC5F2	40S ribosome protein S10	0.85	0.57	0.45	0.76	0.67	Rps10a
NCU06743.1	SC2A4	40S ribosome protein S10	0.62	0.62	0.48	0.76	0.61	Rps10a
NCU06892.1	W09F11	40s ribosomal protein s20	0.75	0.61	0.4	0.87	0.85	Rps20
NCU07408.1	NM5H3	60S ribosomal protein P0	0.56	0.7	0.44	0.8	0.76	Rpp0
NCU07420.1	NC1D6	Translation initiation factor eIF-4A	0.41	0.88	0.39	1.1	0.51	Tif1
NCU07420.1	NP5E5	Translation initiation factor eIF-4A	0.37	0.62	0.37	0.94	0.57	Tif1

Table 3.6 (continued)

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	*Sce* Homolog
NCU07420.1	W08D5	translation initiation factor eIF-4A	0.92	1.1	0.56	1.1	0.45	Tif1
NCU07700.1	NC3H9	elongation factor 2	2	0.91	0.35	0.98	0.3	Eft2
NCU07831.1	NM7B1	translation initiation factor eif-3	0.96	0.54	0.87	0.95	1.2	Nip1
NCU08389.1	W10G5	ribosomal protein L18a	0.24	0.23	0.12	0.17	0.07	Rpl20a
NCU08500.1	W06D7	ribosomal protein Rps8bp	0.72	0.5	0.45	0.76	0.69	Rps8a
NCU08627.1	W09G3	ribosomal protein S21	0.97	0.5	0.41	0.91	0.67	Rps21b
NON	60S R	ribosomal protein	0.99	0.38	0.54	0.83	0.92	RPS21B
	40S 1/4	ribosomal protein	0.81	0.61	0.38	0.78	0.57	NH [b]
	40S-RP	ribosomal protein	0.82	0.73	0.43	0.98	0.77	NH
NCU07002.1	NM9E12	RLR1 - regulatory protein	0.8	0.62	0.55	0.68	0.46	Rlr1
Cytoskeleton								Homolog
NCU00202.1	W13D12	Coronin	0.34	0.79	0.32	0.87	0.38	Crn1
NCU01204.1	NC4E9	tropomyosin	0.43	0.58	0.68	0.62	0.85	Tpm1
NCU01587.1	NM4B4	related to cofilin (actin binding)	0.47	0.6	0.72	0.76	0.96	Cof1
NCU03992.1	SC2E6	fibrim (actin filament bundling protein)	0.76	0.63	0.88	0.79	0.97	Sac6
NCU04054.1	NC3D5	tubulin beta chain	0.65	0.48	0.48	0.97	0.84	Tub2
NCU04173.1	W17E2	Actin	0.34	0.64	0.78	0.64	0.85	Act1
NCU04173.1	ACTIN	Actin	0.31	0.39	0.58	0.62	0.74	Act1
NCU08068.1	NC3B2	spindle pole body protein	0.66	0.63	0.57	0.76	0.49	Spc19
NCU09468.1	NP6G3	alpha-tubulin	0.49	0.84	0.49	0.87	0.61	Tub1
NCU09488.1	SC1F8	protein for organization of actin cytoskeleton	1.1	0.71	0.32	0.43	0.36	Sda1
NCU09488.1	SC2E4	required for organization of actin	0.93	0.29	0.11	0.25	0.091	Sda1
NCU09572.1	NC3A1	ARP2/3 control of actin polymerization	1.3	0.63	0.31	0.94	0.52	Arc18
NCU09700.1	NM2C7	t-complex protein 1	0.14	0.47	0.15	0.31	0.19	Cct7
Nucleotide Synthesis								
NCU06110.1	NC3B5	THI4 - thiamine biosynthesis	0.95	0.41	0.22	0.43	0.21	Thi4

Table 3.6 (continued)

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	*Sce* Homolog
NCU06110.1	NP3F8	THI4 - thiamine biosynthesis	0.89	0.38	0.19	0.34	0.17	Thi4
NCU09345.1	NC4D9	Thiamine biosynthesis protein NMT-1	0.94	0.19	0.11	0.29	0.08	Thi13
NCU09345.1	NMT-1	Thiamine biosynthesis protein NMT-1	1	0.35	0.17	0.4	0.16	Thi13
NCU09345.1	SC5F6	Thiamine biosynthesis protein NMT-1	0.84	0.23	0.12	0.26	0.14	Thi13
NCU09345.1	W01E6	Thiamine biosynthesis protein NMT-1	0.91	0.39	0.38	0.47	0.44	Thi13
NCU09345.1	W06B10	Thiamine biosynthesis protein NMT-1	0.85	0.34	0.13	0.39	0.1	Thi13
NCU09345.1	W07C10	Thiamine biosynthesis protein NMT-1	0.94	0.26	0.1	0.26	0.07	Thi13
NCU09345.1	W09C3	Thiamine biosynthesis protein NMT-1	0.82	0.25	0.11	0.27	0.08	Thi13
NCU09345.1	W10E9	Thiamine biosynthesis protein NMT-1	1	0.24	0.12	0.33	0.1	Thi13
NCU06673.1	NM5B2	dCMP deaminase	0.43	0.76	0.67	0.94	0.35	Dcd1
NCU06673.1	W15E5	dCMP deaminase	0.42	0.94	0.52	0.91	0.25	Dcd1
Carbohydrate Synthase								
NCU06020.1	W10A7	extracellular chitinase	0.61	0.54	0.33	0.6	0.52	Ydr371w
NCU06871.1	SC5H10	glucan synthase	0.71	0.37	0.21	0.29	0.21	Gsc2
NCU00793.1	SC3H8	alpha-trehalose-phosphate synthase	1.8	2.21	1.98	2.58	2.05	Tps3
NCU05127.1	SC1G6	D-alanine--D-alanine ligase	2.4	0.71	1.3	0.75	1.3	NH
Vitamins, Cofactor Biosynthesis								
NCU01271.1	NC1F9	ornithine decarboxylase	0.6	0.74	0.45	1.14	0.5	Spe1
NCU07774.1	NC1B12	GTP cyclohydrolase	0.81	0.92	0.61	1	0.46	Fol2
NCU06549.1	NM1F2	Sno-type pyridoxine vitamin B6	0.89	0.45	0.31	0.56	0.31	Sno2
NCU03500.1	NP6F5	Aminotransferase	0.42	0.99	0.49	0.92	0.56	Bna3
NCU03188.1	NM2H1	D-arabinono-1,4-lactone oxidase mins	1.28	2.48	4.8	1.62	4.32	Alo1
NCU06550.1	SC7F11	Snz-type pyridoxine vitamin B6 biosynthetic	0.59	0.46	0.17	1	0.18	Snz1

[a] The columns in this table are the same as Table 3.1.
[b] NH indicates that the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*

Table 3.7 *N. crassa* genes involved in general degradation.

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	*Sce* Homolog
Protein degradation								
NCU00651.1	SC1A9	peptide N-glycanase	0.26	0.6	0.48	0.57	0.48	Png1
NCU00673.1	NM5A11	subtilisin-like serine protease	1.98	1.18	5.08	1.39	3.74	Prb1
NCU00673.1	NM5D6	subtilisin-like serine protease	1.84	1.27	4.57	1.69	3.52	Prb1
NCU04370.1	SP6F9	ubiquitin-activating enzyme	0.97	1.81	1.32	1.66	1.16	Uba1
NCU05592.1	NM7D1	ubiquitin--conjugating enzyme	1.81	1.37	2.49	1.13	1.8	Ubc6
NCU10046.1	NM3E1	ubiquitin conjugating enzyme	2.29	1.67	1.93	1.24	1.85	Ubc8
NCU00477.1	NM1E4	Serine carboxypeptidase	3.88	2.65	3.72	1.47	2.86	Prc1
NCU02059.1	W07A10	aspartic proteinase	0.87	0.68	0.42	0.81	0.4	Pep4
NCU02549.1	W06E1	Mitochondrial processing peptidase	1.5	0.83	0.33	1.09	0.43	Mas1
Alternative Carbohydrate Degradation								
NCU07027.1	NC5C12	glycogen phosphorylase	1.14	0.65	1.65	1.23	2.1	Gph1
NCU07027.1	NM6E2	glycogen phosphorylase	1.09	0.67	1.77	1.25	2.09	Gph1
NCU03731.1	NM5C6	hydrolase	0.76	1.54	3.15	1.25	4.18	NH [b]
NCU03731.1	W01F8	hydrolase	1.3	1.76	3.38	1.08	4.19	NH
NCU03530.1	W07E6	chitinase 3	1.45	4.41	1.76	2.38	1.35	NH
NCU04952.1	NM5F8	beta-D-glucoside glucohydrolase	1.31	1.36	4.07	1.02	2.97	NH
NCU04952.1	NP3B6	beta-D-glucoside glucohydrolase	1.27	1.17	4.38	0.97	3.61	NH
NCU04952.1	SM2B1	beta-D-glucoside glucohydrolase	0.99	1.23	4.91	0.8	2.63	NH
NCU00061.1	NM7H10	endo 1-3,1-4-beta-glucanase	1.89	3.83	3.57	2.02	2.25	Crr1
NCU04850.1	NP6E3	Beta-1,3 exoglucanase	1.26	1	1	0.49	0.86	NH
NCU05429.1	NC1E1	1,4-glucan branching enzyme	0.52	2.62	1.96	2.16	2.74	Glc3
NCU01517.1	NM6B8	Glucan 1,4-alpha-glucosidase	14.3	16.6	26.5	5.61	13.7	Sga1
NCU09326.1	NC5H6	beta-glucosidase 5	1.32	0.58	0.37	1	0.6	Scw10
NCU06005.1	NM1C7	Glycerol kinase	2.22	1.81	2.27	2.26	2.7	Gut1

Table 3.7 (continued)

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	*Sce* Homolog
NCU02583.1	NM1E6	alpha-glucosidase	2.1	1.5	1.1	1.6	1	Rot2
NCU08332.1	NC1F8	V-ATPase	0.59	0.83	0.39	1.23	0.42	HYP2
NCU08332.1	SP4G12	V-ATPase	0.62	0.81	0.38	1.28	0.42	Anb1
UNKNOWN	W10E7	ubiquitin C-terminal hydrolase	0.74	0.53	0.38	0.72	0.67	NH
NCU07318.1	SC2A10	Mannitol-1-phosphate 5-dehydrogenase	0.49	0.58	0.38	0.77	0.49	Yel070w
NCU07318.1	SC5A11	Mannitol-1-phosphate 5-dehydrogenase	0.65	0.48	0.4	0.76	0.54	Yel070w
NCU07318.1	SC2F11	Mannitol-1-phosphate 5-dehydrogenase	0.26	0.78	0.25	0.9	0.23	Yel070w
Nucleotide Degradation								
NCU09659.1	NM2D1	5'-nucleotidase	0.33	0.82	0.35	0.64	0.32	NH
NCU01939.1	W17B2	RanBP7/importin-beta/Cse1p	1.6	2	3.1	1.6	1.9	Nmd5p
NCU00548.1	W17C9	sugar-nucleotide hydrolase	0.86	0.67	0.45	0.86	0.48	Ysa1
NCU00843.1	W17G1	phosphoribosylglycinamide formyltransferase	0.93	0.56	0.44	0.81	0.38	Ade8
NCU03117.1	W07E10	inosine-5-monophosphate dehydrogenase	0.49	0.68	0.41	1	0.64	Imd4

[a] The columns in this table are the same as Table 3.1.
[b] NH indicates that the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*

and nucleotide biosynthesis display decreased transcription in *rco-3* and *dgr-1* mutants (Table 3.6). One example is provided by the thiamine biosynthetic genes, which were repressed by thiamine in *S. cerevisiae*. Multiple ESTs representing two thiamine biosynthetic genes (*thi4* and *thi13*) are present on the array. Both *thi4* and *thi13* homologs are constitutively expressed in the wild type strain but their expression is dramatically reduced in *rco-3* and *dgr-1* mutants (Table 3.6). Glucose deprivation further repressed their expression (Table 3.6). Therefore, *rco-3* and *dgr-1* may regulate thiamine repressible genes independent of glucose regulation. Again, this finding indicates that *rco-3* and *dgr-1* do not solely influence sugar transport or glucose regulated genes.

In contrast to the general repression of biosynthetic genes, many transcripts involved in general degradation, such as alternative carbon utilization, ubiquitin-dependent and proteasome-dependent protein degradation, display elevated expression in *rco-3* and *dgr-1* mutants, reflecting an increased need to utilize existing nutrient sources to provide fuel for cell growth (Table 3.7). One example is that the transcript encoding glucan 1,4-alpha-glucosidase, a gene involved in glucan utilization, is induced more than six fold in *rco-3* and *dgr-1* mutants even in the presence of glucose (Table 3.7). And several polysaccharide degradation enzymes are expressed at high level during glucose starvation in *rco-3* and *dgr-1* strains (Table 3.7), which may reflect a developmental regulation of cell wall remodeling.

Signal transduction

Signal transduction pathways play an essential role in linking the cellular events to external environmental stimuli in eukaryotic cell. My model is that *rco-3* functions as a sugar sensor to sense extracellular nutrient and transduces a signal to regulate various physiological responses as described above. A variety of genes involved in signal transduction and transcriptional regulation were found to be regulated by *rco-3* and *dgr-1*. These may be the potential components or targets of the putative *rco-3* signaling pathway. Yeast Hsl7 homolog is highly induced in *rco-3* and *dgr-1* mutants and is further stimulated upon glucose deprivation (Table 3.8). Hsl7 encodes a protein methyltransferase that negatively regulates the filamentation/mating mitogen-activated protein (MAP) kinase pathway in *S. cerevisiae* (McMillan *et al.,* 1999). Thus, elevated expression of Hsl7 homolog implicates a down regulation of the MAP kinase cascade. I do observe that several *N. crassa* MAP kinase genes display slightly lower expression in *rco-3* and *dgr-1* mutants (Table 3.8), such as Slt2 homolog (cell wall integrity pathway), Fus3 homolog (mating-pheromone response pathway) and Hog1 homolog (osmolarity pathway) (Gustin *et al.,* 1998). This Hsl7 homolog has been disrupted in *N. crassa* and causes female fertility and colony morphology defects similar to the knockout of Fus3 homolog (Bobrowicz and Ebbole, personal communication). These findings suggest that components of the mating pathway may be down regulated by glucose starvation or induction of asexual development (conidiation) in *N. crassa*.

Table 3.8 *N. crassa* genes involved in signal transduction.

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	*Sce* Homolog
Signal Transduction								
NCU01613.1	SP1F10	Shk1 kinase-binding protein 1	14	13	27	4.3	14	Hsl7
NCU06230.1	NM5D12	Serine/threonine kinase	1.2	1.4	2.2	1.7	4.9	Ksp1
NCU00625.1	NM4H10	UVSB PI-3 kinase	3.15	2.1	3.8	1.9	3	Srp101
NCU08377.1	NP2C2	Adenylate cyclase	0.95	1.8	8.3	2.1	6.1	Ptc1
NCU01569.1	SM2D7	G-beta like protein (WD repeat protein)	7.7	7.2	16	3.2	9.1	Asc1
NCU02463.1	SM2A6	Putative receptor-associated protein	3.2	1.9	2.5	1.7	3.2	Ynl305c
NCU08331.1	NM5G4	Putative coiled coil protein	1.9	1.4	2.8	1.1	2.6	Uso1
NCU02801.1	W08E3	peripheral-type benzodiazepine receptor	3.4	0.8	1.8	0.7	1.9	NH [b]
NCU09842.1	SC2D11	MAP kinase	0.25	0.64	0.39	1.2	0.78	Slt2
NCU09842.1	NC4D2	MAP kinase	0.44	0.72	0.77	0.92	0.89	Slt2
NCU05353.1	NP3A9	FUS3 MAP kinase	1.9	0.84	0.44	0.95	0.58	Fus3
NCU07024.1	W08A7	Osmotic sensitive-2	0.79	0.44	1.8	0.6	0.38	Hog1
NCU02500.1	NC1H2	pheromone precursor gene (ppg1)	0.81	0.48	0.44	0.65	0.59	NH
NCU06544.1	SC2C1	Protein kinase C (cAMP mediated)	0.68	0.65	0.5	0.8	0.47	Pkc1
NCU06249.1	W13G8	Serine/threonine protein kinase	0.86	0.46	0.086	0.25	0.055	yol045w
NCU03436.1	W06A6	Ser/thr protein phosphatase	0.86	0.85	0.28	0.52	0.42	Sit4
NCU00685.1	NM9C11	Ser/thr/tyr protein kinase	0.83	0.61	0.45	0.6	0.42	Hrr25
NCU05288.1	NC5F1	GDP dissociation inhibitor	0.6	0.47	0.85	0.66	1	Gdi1
NCU00443.1	SC5C8	Spi1-GTP-binding protein	0.44	0.65	0.79	0.76	1.3	Yrb1
NCU00443.1	W07A9	Spi1-GTP-binding protein	0.43	0.58	0.75	0.74	0.93	Yrb1
NCU08923.1	NM5F11	DNA-binding protein (Ras/cAMP pathway)	0.31	0.74	0.25	0.9	0.31	Gis2

Table 3.8 (continued)

Ncr ORF [a]	EST ID	Blast Match	74AG-	RG+	RG-	DG+	DG-	*Sce* Homolog
Transcription Factor								
NCU02934.1	NM1G10	Transcription activator (acu-15)	1.2	1.4	2.6	1.2	3.3	Cat8
NCU06205.1	NP2B9	Transcriptional repressor (rco-1)	0.56	0.56	0.46	0.88	0.76	Tup1
NCU03148.1	W08B1	Transcription factor BTF3	0.56	0.52	0.38	0.75	0.54	Egd1
NCU06186.1	W06D6	Cys2-His2 zinc finger protein	0.71	0.39	0.1	0.23	0.1	Pzf1
NCU08295.1	NM2G11	RNA-binding protein	0.75	0.76	0.4	1.2	0.63	Lhp1
NCU08899.1	NM2A9	transcriptional activator	0.6	0.57	0.32	0.71	0.54	Dal81
NCU07418.1	NM9D4	zinc finger protein	1.6	0.72	2.2	0.5	1.5	NH
NCU03048.1	NM5C2	hypothetical zinc-finger protein	3.5	1.7	4.4	1.4	3.2	Yth1
NCU01357.1	NM2E9	pall related protein	1.4	1.4	3.6	0.91	2	Tos7
NCU02307.1	W08F7	Transcription regulator(arginine-responsive)	0.96	0.96	3.3	0.81	3.3	Arg81
NCU04050.1	NM8A7	cross-pathway control (cpc-1)	0.43	0.83	1.1	1.5	1.1	Gcn4
NCU04050.1	SM2D1	cross-pathway control (cpc-1)	0.47	0.91	1.3	1.6	1.3	Gcn4
NCU04050.1	NC5F9	cross-pathway control (cpc-1)	0.82	1.1	1.2	0.51	0.42	Gcn4
NCU05810.1	W10F8	cross-pathway control (cpc-2)	0.92	0.26	0.24	0.52	0.47	Cpc2

[a] The columns in this table are the same as Table 3.1.
[b] NH indicates that the corresponding *N. crassa* ORF has no homolog in *S. cerevisiae*.

It has been demonstrated that G proteins can regulate the MAP kinase pathways *in S. cerevisiae* (Gustin *et al.,* 1998). Several transcripts encoding genes involved in the G-protein signaling pathway, such as G beta like protein, GDP dissociation inhibitor and Sip-GTP binding protein, are differentially regulated in *rco-3* and *dgr-1* mutants (Table 3.8). Heterotrimeric G proteins can transmit external signals to its effectors to regulate different aspects of cellular physiology. Such effectors include adenylate cyclase, phospholipase and serine/threonine protein kinase (Hamm, 1998). In fact, the expression of adenylate cyclase displays a two fold increase in *rco-3* and *dgr-1* and more than six-fold increase upon glucose starvation (Table 3.8), suggesting an increased cAMP level in *rco-3* and *dgr-1* strains. In addition, several protein kinase and phosphates has altered expression in the *rco-3* and *dgr-1* mutants (Table 3.8), which can affect different aspects of cellular response. Furthermore, several transcription factor and zinc finger proteins, including *cpc-1*, *cpc-2, acu15*, *rco-1*, are differentially regulated in the *rco-3* and *dgr-1* mutants. Taken together, the regulation by *rco-3* and *dgr-1* of genes involved in the MAP kinase pathway, cAMP production, protein kinase/phosphatase, and transcription factors may account for the pleiotropic phenotypes of the *rco-3* and *dgr-1* mutants.

DISCUSSION

Carbon metabolism and conidiation

Development and metabolism have been thought to be intimately related in *N. crassa* and there have been a number of views of the relationship between carbon metabolism and conidiation. It has been noted that cultures grown in media with a

preferred carbon (glucose), and preferred nitrogen (ammonium) source promote glycolysis/fermentation and favor mycelial growth while repressing conidiation, whereas media with low or poor carbon or nitrogen sources promote respiration/gluconeogenesis and stimulate conidiation. However, it is not clear if carbon metabolic flux serves as a signal for development or developmental induction gives a signal to alter metabolism. Since *rco-3* and *dgr-1* mutants are derepressed for conidiation in glucose medium and have reduced sugar transport activities compared to wild type strains, I sought to explore the relationship between carbon metabolism and development to address this question. Therefore, analysis of gene expression in these mutants should help to determine if *rco-3* and *dgr-1* simply affect sugar transport and thereby induce conidiation indirectly or whether they might regulate carbon metabolism and conidiation more directly.

My data suggest that *rco-3* and *dgr-1* induce conidiation by a mechanism that is at least partially independent of carbon starvation. Several observations support this view. First, *rco-3* and *dgr-1* induce conidiation in the presence of glucose. Although *rco-3* and *dgr-1* have reduced sugar transport activity, they grow as fast as wild type strain on solid media or in liquid medium in the presence of peptone (which suppresses conidiation) suggesting that while intracellular glucose levels may be lower in *rco-3* and *dgr-1* mutants, it is still high enough to support maximal growth (Madi *et al.,* 1997). Although in the liquid minimal medium *rco-3* and *dgr-1* show reduced biomass accumulation, this appears to be a developmental effect of induced conidiation rather than an effect of carbon limitation. Second, *rco-3* and *dgr-1* induced the known conidiation-specific genes present on the microarray to a much higher level than in

glucose-starved wild type cells. Glucose starvation alone affects expression of a subset of developmentally regulated genes in wild type cells. For example, *con-10* is known to be independently regulated by glucose and conidiation (Lee and Ebbole, 1998a). The stronger and more rapid induction of conidiation-specific genes observed in the *rco-3* and *dgr-1* mutants indicates glucose-independent regulation of conidiation-specific genes.

The lower expression of genes encoding glycolytic enzymes in *rco-3* and *dgr-1* in the presence of glucose is consistent with a lack of glucose transport or glucose sensing that mimics the response of glucose limitation/starvation in wild-type cells. However, genes involved in gluconeogenesis, including the glyoxylate cycle and part of the TCA cycle, are significantly induced when wild type cells are starved for glucose (Chapter II) whereas these genes are largely unresponsive to glucose levels in the two mutants. Hence, the effect of the mutations on regulation of these pathways of central metabolism does not mimic an effect of low glucose transport or the absence of glucose sensing; rather, the mechanism of gene regulation in response to glucose levels appears to be disrupted. To reiterate, I observe constitutive and partially derepressed phosphoenoylpyruvate carboxykinase expression in *rco-3* and *dgr-1* mutants (Table 3.1) (Figure 3.3) and therefore I conclude that repression and induction of gluconeogenesis in response to carbon status are lost in these mutants.

rco-3 and dgr-1 regulate a common set of genes

The phenotypic similarities of the *rco-3* and *dgr-1* mutants suggested that these genes may be part of a common pathway for regulating sugar transport, carbon

regulation of gene expression and conidiation. If so, they would be expected to have similar effects on gene expression. I used a common RNA as one sample for all array experiments to directly compare each condition to the wild type strain grown in the presence of glucose. This allows me to qualitatively make comparisons between the *rco-3* and *dgr-1* profiles. My microarray data show that *rco-3* and *dgr-1* mutants display nearly identical expression profiles, strongly supporting the hypothesis that these genes comprise part of a common regulatory circuit. However, I should note that there are apparent quantitative differences in the extent to which genes may be affected by the two mutations. Although this is suggested by the microarray data directly, it is more clearly demonstrated by northern blot experiments where exposure times can be optimized for individual genes. For example, NCU10021.1 encode a high-affinity glucose transporter in *N. crassa* (Chapter IV) and its expression is not detected in the presence of glucose in the wild type (Table 3.5) (Figure 3.4). The *rco-3* and *dgr-1* mutants display higher levels of NCU10021.1 mRNA regardless of glucose status compared to wild type glucose grown cells (Table 3.5) (Figure 3.4). However, *dgr-1* displays a two-fold greater induction of NCU10021.1 than *rco-3* based on my microarray analysis. Northern blot results are consistent with this analysis (Figure 3.4). In addition, the NUC09873.1 and *grg-1* genes display elevated expression in *rco-3* and *dgr-1* mutants relative to wild type cells grown in glucose (Table 3.4). But, it appears that *rco-3* and *dgr-1* display somewhat different patterns of regulation of these genes (Figure 3.4). However, in this report, I focused on the expression pattern of genes that are differentially expressed in

rco-3 and *dgr-1* relative to wild type glucose grown cell, rather than the differences between *rco-3* and *dgr-1*, which are indistinguishable for most genes.

rco-3 and dgr-1, sugar transport, and gene regulation

rco-3 and *dgr-1* mutants have altered sugar transport activities relative to wild type. A putative high-affinity glucose transporter NCU10021.1, was not induced in *rco-3* or *dgr-1* mutants under glucose starvation, consistent with measurements of glucose transport activity. Therefore, *rco-3* and *dgr-1* appear to be defective in both in regulation of sugar transporter expression. HXT hexose transporter genes are directly regulated by SNF3 and RGT2 (two glucose sensors), and the glucose repression defects of *Snf3*; *rgt2* double mutants is indirect and is due to the glucose uptake defect of *Snf3*; *rgt2* double mutants (Özcan, 2002). The *rco-3* mutant is very similar to *Snf3*; *rgt2* double mutant in that the *rco-3* mutant is defective in both glucose transporter regulation and carbon regulation of gene expression. Thus, the gene regulation defect in *rco-3* and *dgr-1* does not simply mimic the expression pattern of genes in cells deprived of glucose. Because *rco-3* is a glucose transporter homolog regulating sugar transporter gene expression, it appears to function as a sugar sensor rather than a transporter, much like *snf3* and *rgt2*. However, *rco-3* function differs from *snf3* and *rgt2* in that these yeast sensors apparently are restricted to regulation of sugar transporters whereas *rco-3* (and *dgr-1*) are required for the glucose regulation mechanism (for example, genes in gluconeogenesis, glyoxylate cycle, TCA cycle) and for induction of asexual sporulation. Therefore, RCO-3 has similar function as SNF3 and RGT2 to be a sugar sensor, but may not be exactly the counterpart of SNF3 and RGT2.

Signal transduction

How might *rco-3* and *dgr-1* affect regulation of such a variety of metabolic pathways as well as asexual development? In my microarray experiments, *rco-3* and *dgr-1* mutants were altered in regulation of a number of signal transduction pathways. Although cAMP is involved in regulation of carbon metabolism, changes in transcript levels for the adenylate cyclase gene are not apparent in wild type cells starved for glucose or during the diauxic shift in *S. cerevisiae*. Changes in adenylate cyclase transcript levels may not be necessary for controlling cAMP levels as activation of adenylate cyclase by other factors, such as heterotrimeric G-proteins, may be sufficient to bring about the required change in cAMP levels. Since adenylate cyclase transcript levels were not regulated by glucose starvation in wild type, the observed increase in adenylate cyclase expression in the presence of glucose (two-fold) in the *rco-3* and *dgr-1* mutants that is further amplified by glucose starvation (8.3- and 6.1-fold) likely is part of the signaling pathway for conidiation. *cr-1* encodes adenylate cyclase in *N. crassa*, and *cr-1* mutants are induced for conidiation but lack formation of the aerial hyphae that normally give rise to conidiophores. Therefore, the induction of adenylate cyclase in *rco-3* and *dgr-1* mutants may reflect an increase in the level of cAMP to promote aerial hyphae formation prior to production of conidia. Aerial hyphae formation in the *cr-1* mutant is restored by exogenous application of 1 mM cAMP (Kays *et al.,* 2000). Another possibility is suggested from the *N. crassa* genome sequence (Galagan *et al.,* 2003) that reveals the existence of genes that might constitute components of an extracellular cAMP signaling pathway that would be expected to require synthesis of

large amounts of cAMP. Direct measurements of intra- and extracellular cAMP levels in *rco-3* and *dgr-1* mutants in response to glucose should help clarify these possibilities.

Heterotrimeric G-protein signaling in *N. crassa* and the role of G-proteins in regulation of adenylate cyclase activity has been characterized (Kays *et al.,* 2000; Yang *et al.,* 2002; Ivey *et al.,* 2002). Several G protein mutants, such as *gna-3*, *gna-1* and *gnb-1*, have phenotypic similarities to *rco-3* and *dgr-1* in that they conidiate in liquid culture. However, the regulation of adenylate cyclase activity through G-protein action is thought to occur at a post-transcriptional level (Ivey *et al.,* 2002). Therefore, the effect of *rco-3* and *dgr-1* mutations on adenylate cyclase transcript levels likely occurs independently of heterotrimeric G-protein signaling.

Additional genes involved in signaling and transcriptional activation were differentially expressed in the mutant strains relative to wild type. These include a serine/threonine kinase (NCU06230.1), WD-repeat protein (NCU01569.1), a C2-H2 Zinc finger protein (NCU06186.1), a homolog of *S. cerevisiae* HSL7, a negative regulator of MAP kinase signaling (NCU01613.1). Therefore *rco-3* and *dgr-1* may regulate a variety of cellular responses through several signal transduction pathways.

A final note of caution is that although more than 90% of the elements on the microarray are correctly assigned it is possible that some gene assignments are in error. Therefore, the transcriptional regulation of *cr-1* and the HSL7 homologs require verification by northern blot analysis.

CHAPTER IV

GENETIC ANALYSIS OF A SUGAR SENSING PATHWAY IN *NEUROSPORA CRASSA*

OVERVIEW

The *rco-3* gene is a regulator of sugar transport and conidiation in *Neurospora crassa*. *rco-3* encodes a member of the sugar transporter family and has been proposed to function as a sugar sensor in *N. crassa*. To identify genes that are functionally related to RCO-3, I selected suppressors of the sorbose resistance phenotype of *rco-3* mutants. Three distinct suppressors were discovered from the screen, designated as *ssr-1* (suppressor of sorbose resistance), *ssr-2* and *ssr-3*. All suppressors suppress both the sorbose resistance and derepression of conidiation in *rco-3*. All three suppressors can restore the induction defect of high affinity glucose uptake in the *rco-3* mutants. *dgr-1* is a previously characterized 2-deoxyglucose resistant mutant that phenotypically resembles *rco-3*, therefore, *dgr-1* may be involved in the putative *rco-3* signaling pathway. Epistatic relationship among *rco-3*, *dgr-1* and the suppressors were carried out by analyzing *rco-3*; *dgr-1*, *ssr-2*; *dgr-1* and *ssr-3*; *dgr-1* double mutants. These analyses indicate that *rco-3* is epistatic to *dgr-1*. In addition, I have identified a high affinity glucose transport gene in *N. crassa*, whose transcription is under the control of glucose, *rco-3* and *dgr-1*.

INTRODUCTION

The orange bread mold *Neurospora crassa* is well known as a useful model for the study of filamentous fungi. In *N. crassa*, macroconidiation (conidiation) is the major asexual reproduction cycle and is subject to regulation by several environmental cues including carbon limitation. Conidiation can be induced in submerged culture in liquid medium lacking carbon or nitrogen source. Glucose is the universal carbon and energy source for most eukaryotic cells including *N. crassa*. Glucose regulation of gene transcription patterns in *N. crassa* has been examined via transcriptional profiling in our laboratory (Chapter II). However, the mechanisms by which fungi sense glucose or other carbon sources to control gene expression and cell differentiation is still unclear.

The *rco-3* (regulator of conidiation gene-3) mutant was originally isolated due to elevated expression of a conidiation specific gene (*con-10*) (Madi *et al.,* 1994). Subsequent cloning and characterization indicate that *rco-3* is involved in regulation of conidiation, sugar transport and carbon repression and was proposed to play a regulatory role rather than act directly as a glucose transporter in *N. crassa* (Madi *et al.,* 1997). Thus, *rco-3* may provide a link between glucose sensing and conidiation in *N. crassa*. Understanding the putative *rco-3* signaling pathway will provide a basis to elucidate the sugar sensing signaling cascades and how glucose limitation triggers conidiation in filamentous fungi.

2-deoxyglucose is a toxic sugar analog. It can be phosphorylated to 2-deoxyglucose-6-phosphate, which can accumulate and inhibit growth of wild type cells (Moore, 1981). A previously isolated 2-deoxyglucose resistant mutant (*dgr-1*) displays

phenotypes similar to *rco-3* (Allen *et al.,* 1989), and *rco-3* is allelic to another 2-deoxyglucose resistant mutant (*dgr-3*) (data not shown). Therefore, *dgr-1* may be involved in the putative *rco-3* signaling pathway.

rco-3 encodes a protein with most sequence similarity to the sugar transporter superfamily and *rco-3* has altered glucose transport activities compared to wild type (Madi *et al.,* 1997). Two glucose transport systems have been characterized in *N. crassa* (Scarborough, 1970; Schneider and Wiley, 1971). One is a constitutive low-affinity transport system with a Km similar to the *S. cerevisiae* low-affinity glucose transport system (Km=25 mM). This system can transport glucose, 3-O-methyl glucose, and L-sorbose. The other is a glucose-repressible high-affinity transport system, which transports glucose, L-sorbose, galactose, 3-O-methyl glucose, mannose and 2-deoxyglucose with a Km of approximately 0.04 mM (Crocken and Tatum, 1967; Schneider and Wiley, 1971). Therefore, the glucose transport defect of *rco-3* mutants might be responsible for the sorbose and 2-deoxyglucose resistance phenotypes. In *S. cerevisiae*, it has been shown that glucose (hexose) transporters can transport both glucose and fructose (Coons *et al.,* 1995). *N. crassa* has been shown to posses a separate glucose-repressible fructose uptake system with a Km of 0.4 mM, and L-sorbose competitively inhibits fructose uptake (Rand and Tatum, 1980).

Suppressor screens have proven to be a very powerful tool to elucidate the glucose signaling pathways in *S. cerevisiae*. *snf3* was first identified by isolating mutants defective in growth on sucrose or raffinose (Neigeborn and Carlson, 1984) and subsequent characterization indicated that *snf3* lacks high affinity glucose uptake but

exhibits normal low affinity glucose transport activity (Bisson *et al.,* 1987). Selection of multicopy and genomic suppressors of *snf3* growth defects led to the identification of hexose transporter genes (Bisson *et al.,* 1987; Kruckeberg and Bisson, 1990), a high glucose concentration sensor, RGT2, and a *snf3*-responsive transcription factor, RGT1, which is responsible for regulation of hexose transporter expression in *S. cerevisiae* (Marshall-Carlson *et al.,* 1993; Özcan *et al.,* 1998; Mosley *et al.,* 2003). Further studies demonstrated that *snf3* encode a sugar sensor rather than a sugar transporter (Özcan *et al.,* 1998). Therefore several central components as well as target genes of the SNF3/RGT2 signaling cascade were discovered from suppressor studies.

In this study, I characterize the sugar transport activities and conidiation phenotypes of *rco-3*, *dgr-1* and the *rco-3*; *dgr-1* double mutant. By selecting mutants that remedy sorbose resistance of *rco-3* mutants, I recovered several suppressors that fell into three complementation groups, designated as *ssr-1* (suppressor of sorbose resistance-1), *ssr-2* and *ssr-3*. As *dgr-1* is likely involved in the putative *rco-3* signaling pathway, I generated *ssr-2*; *dgr-1* and *ssr-3*; *dgr-1* double mutants and characterized these double mutants. This analysis indicates that these suppressors partially suppress the *dgr-1* phenotypes.

In addition, I examined the expression pattern of several genes that participated in sugar transport and gluconeogenesis, as well as glucose repressible genes, in wild type, *rco-3*, *dgr-1* and *rco-3*; *dgr-1* strains in response to glucose and peptone. Northern blot analysis revealed that *rco-3* and *dgr-1* are generally defective in carbon repression, which is consistent with microarray analysis (Chapter III). A cDNA clone showing

sequence similarity to yeast hexose transporters is differentially regulated by glucose and its regulation is altered in *rco-3* and *dgr-1* mutants from microarray experiment and Northern blot analysis. Disruption and overexpression of this gene demonstrates that it encodes a high affinity glucose transporter in *N. crassa*. Taken together, my studies identified several new components of the putative *rco-3* signaling cascade as well as target genes of the pathway.

MATERIALS AND METHODS

Strains and growth conditions

Unless otherwise noted, strains used in this study were obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas, Kansas City, Kansas, 66160-7420. Strain Oak Ridge 74OR23-1A (FGSC no. 987) and ORS-6a (FGSC no. 2490) are wild type strains. *rco-3* (null mutant generated by RIP) (Madi *et al.,* 1997) and *dgr-1* (FGSC no. 4326) were used in this study. A cross of *rco-3* and *dgr-1* mutant strains was performed to obtain *rco-3*; *dgr-1* double mutant. The genotype of putative double mutants was verified by backcrossing to the wild type strain.

Cultures were grown in Vogel's minimal medium (Davis and De Serres, 1970) supplemented with glucose as described previously (Madi *et al.,* 1997). Conidia were collected with sterile water through Miracloth (Calbiochem) from a 50 ml solid medium culture after six days of growth. Conidia were counted with a hemocytometer (Hausser Scientific) and inoculated at 10^6 conidia / ml into 50 ml liquid minimum medium containing 2% peptone and 2% glucose. Cultures were grown approximately 17-18

hours on a rotary shaker (200 rpm) at 34°C. The mycelium was harvested by vacuum filtration on sterile Whatman filter paper and cut with a sterile razor blade into two pieces of approximately equal size. The halves of each mycelial pad were inoculated in parallel into 50 ml fresh 2% glucose-supplemented minimal medium (glucose repression) or the same medium lacking glucose (glucose derepression). Since peptone can suppress the derepression of conidiation in *rco-3* and *dgr-1* strains, but not sugar transport defects (data not shown), I also inoculate the mycelium pad into peptone containing medium supplemented with glucose (glucose repression) or without glucose (glucose derepression). The inoculated flasks were vigorously shaken to disperse the cells and incubated for two hours at 34°C. Cultures were harvested by filtration and immediately used for sugar transport assay or frozen in liquid nitrogen prior to RNA extraction.

Mutant isolation and genetic technique

To isolate suppressors of the *rco-3* mutants, conidia from *rco-3* strain were mutagenized by exposure to UV light to approximately 50% killing and plated on 2% sorbose containing medium (Davis and De Serres, 1970). Sorbose sensitive mutants were isolated and purified to homokaryon by serial transfer. Fourteen isolates were confirmed to contain single mutation through backcrossing to wild type strains. Three distinct suppressors were isolated through complementation testing, designated as *ssr-1*, *ssr-2*, *ssr-3*. Auxotrophic suppressor strains were generated by crossing suppressors to *rco-3* strains containing auxotrophic markers. Heterokaryon tests were performed by fusing auxotrophic suppressor strains with *rco-3* containing another nutrient marker. To

isolate double mutants of each suppressor also containing *dgr-1* mutation, *ssr*; *rco-3* was crossed to *dgr-1* and progeny were scored for sorbose resistance and mating type as described. For sorbose resistance, they were examined for colony morphology (spreading vs dense colony) on Vogel medium containing 0.05% glucose, 0.05% fructose and 2% L-sorbose. Since *rco-3* gene maps 5 cM from the mating type locus (MAT) (Madi *et al.,* 1997), I used MAT locus as a co-segregating marker for the *rco-3* gene. Wild type stains were inoculated on low nitrogen-containing synthetic cross (SC) plates and grown for seven days. Aerial hyphae were removed with a sterile inoculation loop and conidia from each of the progeny were added to SC plates containing protoperithecia of wild type. Four days after inoculation, perithecia observed on the surface of the plates revealed the mating type of each of the progeny. Crosses were performed similarly using SC slants instead of SC plates. After mature perithecia released ascospores, random spores were picked up and germinated as previously described (Perkins and Raju, 1986).

Nucleic acid isolation, hybridization and sequencing

Genomic DNA was isolated as previously described (Vollmer and Yanofsky, 1986). RNA extraction and Northern blot hybridization were performed following the protocol described previously (Madi *et al.,* 1994). DNA probes for NCU10021.1 (cDNA clone *SP1A4*), NCU06358.1 (cDNA clone NP4E6), NCU09873.1 (phosphoenoylpyruvate carboxykinase), and *grg-1* were obtained from the corresponding sequence-verified EST clones by PCR. RNA quality and loading were verified by visual inspection of rRNA band intensity and hybridization with an rDNA

probe. Sequencing of cDNA clones was performed using the Prism dideoxy sequencing kit (Applied Biosystem) with T3 and T7 primers in the Gene Technology Laboratory (Texas A&M University).

Sugar uptake experiments

Sugar transport assays were performed as described previously (Madi *et al.,* 1997). Wild type, *rco-3* and *dgr-1* strains were cultured as described above to obtain glucose repressed and derepressed cells. The sugar transport experiments were initiated by adding D-[U-^{14}C] glucose (Amersham Life Science) or D-[U-^{14}C] fructose (Amersham Life Science) to 125-ml flasks containing 10 ml Vogel minimal salts with either 0.25 mM glucose, 40 mM glucose or 10 mM fructose. A 10 ul of the control sample was removed before and after the sugar transport assay to quantitate the amount of radioactivity and glucose in the medium. The prepared glucose repressed or derepressed cells were added into the flasks and were vigorously shaken to disperse the mycelium. The samples were taken by removing 3 ml cell suspension at the appropriate time intervals. Three time points were taken for each sugar concentration. The cells were washed, dried, weighed and added to scintillation vials to count radioactivity. Sugar uptake per mg mycelium was plotted vs time. The assay was linear over the time period of the experiment and the correlation coefficient (r^2) for linearity was calculated for each assay.

Plasmid construction and transformation

An EST clone (*SP1A4* NCU100021.1) was amplified by PCR and cloned into pSM565 to create overexpression plasmid pX12. pSM565 is a vector with ampicillin

resistance for bacterial selection and hygromycin resistance for selection in fungi. In addition, pSM565 vector possesses RP27 (ribosomal protein 27 from *M. grisea*) promoter, which is highly constitutively expressed in *N. crassa*. Primers were designed as follows: for the 5' primer: 15 base pair (bp) complementary to RP27 promoter sequence + ATG + 15 bp matching the coding region of *SP1A4*. For the 3' primer: 15 bp homologous to the terminator sequence of pSM565 + 15 bp homologous to *SP1A4* sequences starting at the predicted stop coden. It should be noted the full sequence of *SP1A4* genes has not been determined. However the N- and C- terminal regions of the predicted coding sequence have homology to sugar transporters. Co-transformation of the *SP1A4* PCR product and linearlized pSM565 vector into yeast cells was performed as described (Bourett *et al.,* 2002). PCR products were cloned into pSM565 vector by yeast homologous recombination (restriction site independent cloning). Yeast transformants were collected and mixed to extract DNA. The mixed DNA was transformed into E. coli to screen for the desired construct, designated pX12. The integrity of pX12 expression vector was verified by restriction enzyme digestion.

pX12 was transformed into 23ORS74-1VA conidia by electroporation as follows. Conidia from 10 to 20 day old cultures were harvested with sterile 1M sorbitol, filtered through sterilized miracloth, washed three times with 1M sorbitol and resuspended at 1.25 X 10^{10} cell /ml in 1M sorbitol. 0.3 to 2.0 µg plasmid DNA was added into 100 µl of the conidial suspension and incubated on ice fore 30 minutes. Electroporation was performed at 1.5 kv, 25 mA and 800 ohms in 0.2 cm cuvette (Bio-Rad Gene Pulser). The transformed conidia were mixed with regeneration agar (Vogel's

salts, 1M sorbitol, 1.5% agar, FGS) and were poured on to sorbose medium with the appropriate selection drug. The plates were incubated at 34°C for 2 to 3 days to obtain single transformants.

For repeat induced point (RIP) mutagenesis (Selker and Garrett, 1988; Selker, 1990). The transformant isolate containing a single copy of pX12 was crossed with ORS-6a. Random ascospores were collected, germinated and grown in Vogel minimal medium. RNA was extracted to examine *SP1A4* transcription via Northern blot. The *SP1A4* RIP mutants were further confirmed by Southern blot analysis.

RESULTS

Characterization of the rco-3, dgr-1and rco-3; dgr-1 mutants

Sorbose restricts radial growth of *N. crassa* causing growth as compact dense colonies on 2% sorbose medium (Figure 4.1), whereas *rco-3* mutants are resistant and grow as somewhat transparent spreading colonies (Figure 4.1). In addition, unlike wild type, *rco-3* mutants form large amounts of aerial hyphae and release mature conidia (Figure 4.1). Characterization of *dgr-1* (2-deoxyglucose resistance-1) mutants indicated that they appear to be slightly less resistant to sorbose than *rco-3* mutants, although they also conidiate on sorbose plates (Figure 4.1). The *dgr-1*; *rco-3* double mutants most closely resemble *rco-3* on sorbose medium (Figure 4.1), indicating that *rco-3* is epistatic to *dgr-1*.

Several environmental cues including glucose limitation cause induction of conidiation in submerged culture. Wild type strains grow primarily as hyphae in

minimum medium with 2% glucose, whereas *rco-3* mutants produce budding cells resembling conidiophores (Figure 4.2). The *dgr-1* mutant displays a similar pattern of growth but generates fewer conidiophores than *rco-3* (Figure 4.2). *rco-3*; *dgr-1* mutants display the same conidiation patterns as *rco-3* strains (Figure 4.2). As previously reported, conidiation of *rco-3* was abolished when peptone was added into the medium (Madi *et al.,* 1997). The induction of asexual development in *dgr-1* and *rco-3*; *dgr-1* strains was also suppressed in peptone containing medium.

Glucose uptake of the wild type, rco-3, dgr-1and rco-3; dgr-1 strains

N. crassa possesses a constitutive low affinity glucose transport system and a glucose repressible high-affinity glucose transport system (Scarborough, 1970; Schneider and Wiley, 1971). In addition, there is a separate glucose-repressible fructose uptake system in *N. crassa* (Rand and Tatum, 1980). To distinguish high- and low-affinity glucose transport activity, I used 0.25 mM glucose, a saturating substrate concentration for the high-affinity glucose transport system in measurements of high affinity glucose transport system. A high concentration of glucose, 40 mM, was used to examine the combined high- and low-affinity glucose transport activities. Since the Km for fructose uptake is 0.4 mM, I measured fructose transport activity using 10 mM fructose as the substrate concentration.

In the glucose-grown wild type strain, there is a very low level of high-affinity glucose transport activity (Table 4.1). Therefore the observed glucose transport at 40 mM glucose mainly reflects the activity of the low affinity glucose transport system (Table 4.1). When cells are starved for glucose for two hours, I observed a significant

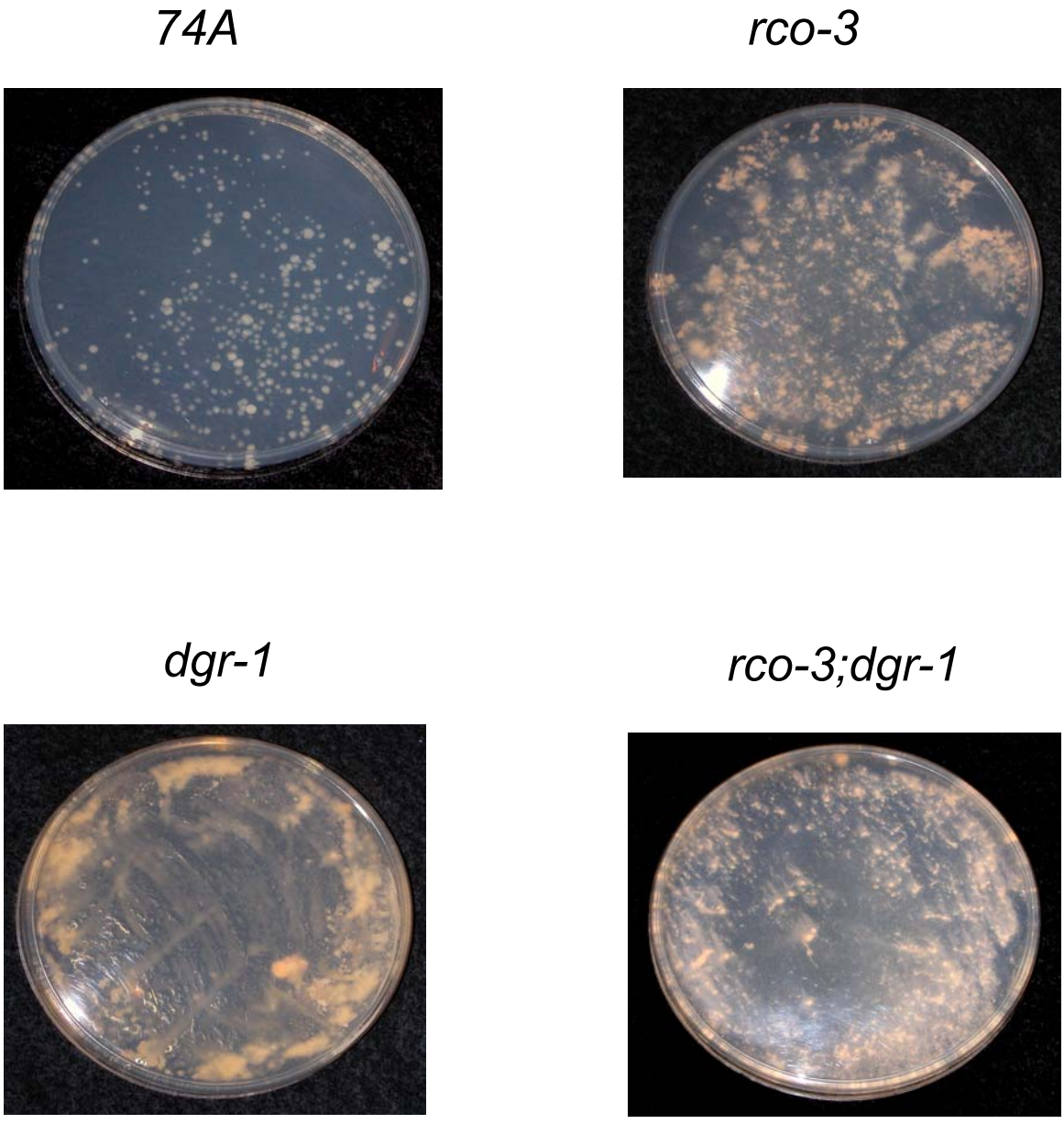


Figure 4.1 Growth properties on sorbose medium. The wild type (*74A*) strain grows as small dense colony on sorbose medium. *rco-3*, *dgr1* and *rco-3; dgr-1* mutants are sorbose resistant and form aerial hyphae on sorbose medium.

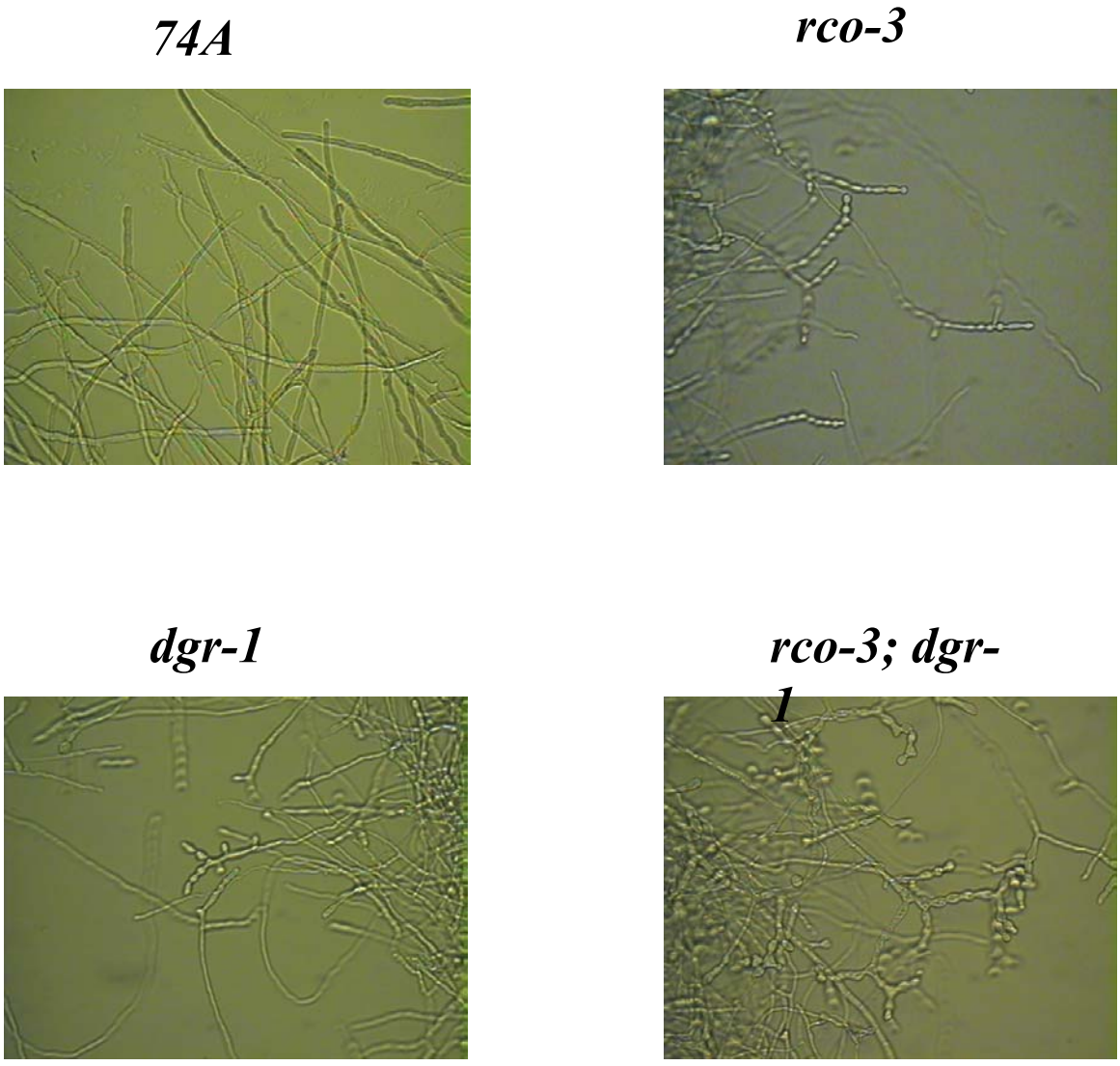


Figure 4.2 Conidiation of wild type (*74A*), *rco-3*, *dgr-1* and *rco-3*; *dgr-1* in liquid medium. Wild type, *rco-3*, *dgr-1* and *rco-3*; *dgr-1* strains were cultured in minimum medium with 2% glucose. *74A* grows primarily as vegetative hyphae in glucose rich medium. *rco-3*, *dgr-1* and *rco-3*; *dgr-1* form conidial chains in submerged culture.

increase in high affinity glucose transport activity as previously described (Table 4.1) (Scarborough, 1970; Schneider and Wiley, 1971). The increased glucose transport activity at 40 mM glucose in glucose starved cells is the sum of uptake by the induced high affinity transport system under glucose derepression and the constitutive low affinity transport activity (Table 4.1). Also, I observed the glucose repressible fructose transport system, in which fructose transport activity increased nine-fold when cell were starved for glucose (Table 4.1).

All three sugar transport activities are significantly altered in the *rco-3* mutants compared to wild type. *rco-3* has an elevated high affinity glucose transport activity in the presence of glucose (Table 4.1), indicating a partial release of glucose repression of the high affinity glucose transport system. However, the elevated high affinity glucose transport activity was not further induced when *rco-3* cells were starved for glucose (Table 4.1). In contrast to elevated high affinity glucose transport activities, *rco-3* possesses a reduced level of low affinity glucose transport activity (Table 4.1). The fructose transport activity is partially derepressed and constitutive in the *rco-3* mutant (Table 4.1).

The *dgr-1* strain displays a pattern of low affinity glucose transport and fructose transport systems similar to *rco-3* in response to glucose status (Table 4.1). However, in the *dgr-1* mutant, the high-affinity glucose transport activity was partially depressed upon glucose limitation (Table 4.1). Thus, the sugar transport measurements demonstrate that *rco-3* and *dgr-1* are defective in the regulation of three independent sugar transport systems in *N. crassa*.

Table 4.1 Glucose and fructose transport in the *74A, rco-3*, *dgr-1* and *rco-3; dgr-1* strains.

	Glucose-grown cells				Glucose-starved cells			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Glucose 0.25 mM	**0.25 (0.99)**	**0.9(0.98)**	**1.4(0.99)**	**0.7(0.99)**	**17(0.99)**	**0.8(0.99)**	**6.4(0.95)**	**1.1(0.99)**
40 mM	**9.5(1)**	**3.8(0.91)**	**3.2(0.99)**	**3.6(0.98)**	**25(0.99)**	**5.2(0.96)**	**8.1(0.99)**	**4.2(0.99)**
Fructose 10 mM	**1.1(0.98)**	**3.3(0.99)**	**2.5(1)**	**5.6(0.99)**	**9.2(0.99)**	**3.6(0.94)**	**3.3(0.98)**	**5.6(0.97)**

Values are nmol glucose transported/min/mg dry mycelia
1 = wild type (*74A)* (r^2), 2 = *rco-3*(r^2), 3 = *dgr-1*(r^2), 4 = *rco-3;dgr-1* (r^2)
r^2 is the correlation coefficient for linear uptake of sugar during the time course

The *rco-3*; *dgr-1* double mutant displays the same regulation pattern for the three sugar transport systems as *rco-3* (Table 4.1). In addition, as shown above, the *rco-3*; *dgr-1* double mutant has the same growth morphology and derepressed conidiation as the *rco-3* mutant. Therefore, the nearly identical phenotypes between *rco-3* and *rco-3*; *dgr-1* double mutants indicate that *rco-3* can mask the phenotype of *dgr-1*. This observation indicates epistasis and suggests that *dgr-1* may act downstream of *rco-3* in the putative *rco-3* signaling pathway.

Isolation and genetic analysis of rco-3 suppressors

To identify genes that are functionally related to *rco-3*, I selected suppressors that restored sorbose sensitivity to the mutant. *rco-3* conidia were mutagenized by UV light and plated on sorbose medium to identify compact sorbose sensitive colonies. Each suppressor strain was crossed to *rco-3* or wild type to confirm their segregation as a single mutation. Fourteen suppressors containing a single gene suppressor mutation were identified for further analysis. To test whether suppressor mutations are dominant or recessive, heterokaryon tests were performed. Each suppressor was crossed to an *rco-3*; *arg-4* strain to obtain *rco-3*; *ssr*; *arg-4* auxotrophic strains. Forced heterokaryons were generated by co-inoculation of *rco-3*; *pyr-4* with *rco-3*; *ssr*; *arg-4* strains on sorbose medium to examine the sorbose resistance phenotype. If the heterokaryon is sorbose sensitive, then the suppressor mutation is dominant; otherwise, the suppressor must be recessive. Thirteen of the fourteen suppressors tested are recessive based on the heterokaryon tests.

To assign suppressors into different complementation groups, each *rco-3*; *ssr* was crossed to *rco-3* to generate *rco-3*; *ssr* strains with opposite mating types. Different *rco-3*; *ssr* suppressor strains were crossed to each other and progenies from each cross were scored for sorbose resistance. If two suppressors are allelic, no sorbose resistant progeny should be obtained. For examing sorbose resistant of progeny, random ascospores were directly spread on sorbose medium to allow screening several hundreds progenies. Four suppressors fell into three complementation groups and the remaining ten strains gave ambiguous results and were not analyzed further. Three distinct suppressor mutations are identified and designated *ssr-1* (suppressor of sorbose resistance), *ssr-2*, and *ssr-3*.

Crossing of *ssr-2; rco-3* and *ssr-3; rco-3* with the *rco-3* strain yielded a 1:1 ratio of sorbose resistant and sensitive progeny. By crossing *ssr-2; rco-3* and *ssr-3; rco-3* to the wild type, 25% of the progenies were sorbose resistant and 75% were sorbose sensitive. The sorbose resistant colonies were *rco-3*; ssr^{+} while the remaining 75% of the progeny were either *rco-*3^{+}; ssr^{+}, *rco-3*; ssr^{-} or *rco-*3^{+}; ssr^{-}. The sorbose sensitive progeny were phenotypically indistinguishable. Therefore *ssr-2* and *ssr-3* have no apparent phenotypes on their own and they segregate independently with *rco-3*. The analysis of *ssr-1* indicated a linkage to *rco-3* of approximately 5 cM. Since *rco-3* maps 5 cM to the right of the MAT locus, I determined that *ssr-1* maps approximately 5 cM to the right of *rco-3* and 10 cM to the right of the MAT locus, close to the centromere of linkage group I.

Characterization of rco-3 suppressors

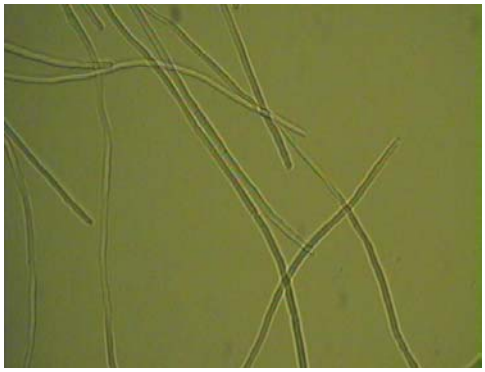
Three suppressors are sorbose sensitive when cultured on sorbose medium (Figure 4.3). To determine whether suppressors also suppress derepressed conidiation of the *rco-3* mutation, conidia of the suppressor strains were cultured in minimal medium containing 2% glucose. All three suppressors grow primarily as filamentous hyphae in submerged cultures as illustrated for *ssr-1*; *rco-3* and *ssr-2*; *rco-3* strains (Figure 4.3), indicating that all three suppressor mutations can suppress the submerged conidiation phenotype of the *rco-3* mutant.

As three suppressors remedy the sorbose resistance phenotype, it is possible that these *ssr* mutations may also restore sugar uptake defects observed in *rco-3* mutants. Therefore, I measured glucose and fructose transport in the three suppressor strains. All three *ssr*; *rco-3* strains display the same high affinity glucose transport activity as the wild type strains in glucose grown cells, indicating these suppressors restore glucose repression of high affinity glucose transport activity (Table 4.2). When derepressed for glucose for two hours, *ssr*; *rco-3* strains were restored for the induction for high affinity glucose transport, although perhaps not the same level as the wild type strain. The low affinity glucose transport was also restored nearly to wild type level in *ssr*; *rco-3* strains Table 4.2). In addition, all three *ssr*; *rco-3* strains completely restore the fructose uptake defect of *rco-3* mutants (Table 4.2).

ssr-1;rco-3

ssr-2;rco-3

ssr-1;rco-3



ssr-2;rco-3

Figure 4.3 Growth studies of *rco-3* suppressors. *ssr-1*; *rco-3* and *ssr-2*; *rco-3* strains form small dense colonies on sorbose medium, indicating *ssr-1* and *ssr-2*suppress sorbose resistance phenotype of *rco-3*. *ssr-1*; *rco-3* and *ssr-2*; *rco-3* grow as filamentous hyphae in Vogel minimum medium with 2% glucose.

Table 4.2 Glucose and fructose transport in the *74A, ssr-1;rco-3*, *ssr-2;rco-3* and *ssr-3;rco-3*strains.

	Glucose-grown cells				Glucose-starved cells			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Glucose 0.25 mM	**0.25[a] (0.99)**	**0.3(0.99)**	**0.2(0.99)**	**0.3(0.99)**	**17(0.99)**	**9.8(0.99)**	**5.2(0.97)**	**9.2(0.99)**
40 mM	**9.5(1)**	**7.8(0.99)**	**5.4(0.99)**	**9.2(0.98)**	**25(0.99)**	**19(0.98)**	**17(0.99)**	**17(0.99)**
Fructose 10 mM	**1.1(0.98)**	**0.3(0.99)**	**0.3(1)**	**0.5(0.9)**	**9.2(0.99)**	**10(0.97)**	**8.5(0.98)**	**12(0.98)**

[a] Values are nmol glucose transported/min/mg dry mycelia
1 = wild type *74A*(r^2), 2 = *ssr-1;rco-3*(r^2), 3 = *ssr-2;rco-3*(r^2), 4 = *ssr-3;rco-3* (r^2)
r^2 is the correlation coefficient for linear uptake during the time course
74A data are taken from previous table for comparison

Isolation and characterization of ssr-2; dgr-1 and ssr-3; dgr-1 strains

The observation that *rco-3* is epistatic to *dgr-1* prompted me to determine whether these *ssr* mutations can suppress the *dgr-1* mutant phenotypes. To test this hypothesis, I crossed *ssr*; *rco-3* strains to the *dgr-1* strain to generate *ssr*; *dgr-1* double mutants. Using the MAT locus as a marker for *rco-3* and examining sorbose resistance in the progeny, I identified putative *ssr*; *dgr-1* double mutants. Potential *ssr*; *dgr-1* strains were back-crossed to confirm their genotypes. Since the *ssr-1* mutation is linked to *rco-3* (5 cM), it was difficult to isolate a *ssr-1*; *dgr-1* double mutant. Therefore, I confined my analysis to *ssr-2*; *dgr-1* and *ssr-3*; *dgr-1* mutants.

ssr-2; *dgr-1* and *ssr-3*; *dgr-1* grow as compact dense colonies on sorbose plates, indicating that *ssr-2* and *ssr-3* suppress the sorbose resistance phenotype of *dgr-1* mutants (data not shown). However, when I examined the sugar transport activities in *ssr-2*; *dgr-1* and *ssr-3*; *dgr-1* strains, both strains display similar transport activities as *dgr-1* in all three sugar uptake systems (Table 4.3), demonstrating that *ssr-2* and *ssr-3* do not suppress the sugar transport defect of *dgr-1* mutants. Thus, my data indicate that all three suppressors at least partially remedy the sugar transport defect of *rco-3* but not *dgr-1* strains. Therefore it is possible that sorbose resistance is not due directly to a sorbose transport defect.

Glucose regulation of gene expression in 74A, rco-3, dgr-1 and rco-3; dgr-1 strains

The transcriptional regulation of several genes that are known to be subjected to carbon regulation were examined in *74A*, *rco-3*, *dgr-1* and *rco-3*; *dgr-1* strains. Each strain was grown in minimal medium with 2% glucose and 2% peptone for 18 hours and

Table 4.3 Glucose and fructose transport in the wild type *74A*, *dgr-1, ssr-2; dgr-1* and *ssr-3; dgr-1* strains.

	Glucose-grown cells				Glucose-starved cells			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Glucose 0.25 mM	**0.25(0.99)**	**1.4(0.99)**	**2.1(0.99)**	**2.1(0.99)**	**17(0.99)**	**6.4(0.95)**	**3.8(0.99)**	**3.2(0.99)**
40 mM	**9.5(1)**	**3.2(0.99)**	**3.6(0.99)**	**4.3(0.99)**	**25(0.99)**	**8.1(0.99)**	**6.3(0.99)**	**6.8(0.98)**
Fructose 10 mM	**1.1(0.98)**	**2.5(1)**	**2(0.98)**	**2.6(0.99)**	**9.2(0.99)**	**3.3(0.98)**	**2.4(1)**	**2.6(0.98)**

Values are nmol glucose transported/min/mg dry mycelia
[a] r^2 is the correlation coefficient for linear uptake during the time course
1 = *74A*(r^2), 2 = *dgr-1*(r^2), 3 = *ssr-2; dgr-1*(r^2), 4 = *ssr-3; dgr-1*(r^2)
74A and *dgr-1* data are taken from previous table for comparison

transferred to medium with 2% glucose (glucose repression) or medium lacking glucose (glucose derepression) for two hours. Since peptone suppresses the submerged conidiation phenotype, I also examined gene regulation in medium with or without peptone in conjunction with the presence and absence of glucose.

NCU10021.1 encodes a high affinity glucose transporter in *N. crassa* (see below). Glucose repression of NCU10021.1 transcription is readily observed in wild type (Figure 4.4). However, the expression of NCU10021.1 is partially derepressed in *rco-3*, *dgr-1* and *rco-3; dgr-1* strains. *rco-3* and *dgr-1* are required for full induction of NCU10021.1. NCU06358.1 is another glucose transporter homolog whose regulation differs from NCU10021.1 (Figure 4.4). In wild type strain, NCU06358.1 mRNA was abundant and its expression decreased somewhat in the absence of glucose. *rco-3*, *dgr-1* and *rco-3*; *dgr-1* strains display similar but more subtle transcription regulation of NCU06358.1 as wild type strain (Figure 4.4). NCU09873.1 encodes a protein with sequence similarity to phosphoenolpyruvate carboxykinase (*PEPCK*), a key enzyme in gluconeogenesis. Glucose apparently represses the expression of *PEPCK* in wild type both in the presence and absence of peptone, but only weakly suppress it in *rco-3, dgr-1* and *rco-3*; *dgr-1* strains in the presence of peptone (Figure 4.4). An interesting observation is that peptone greatly stimulates the expression of *PEPCK* in all strain backgrounds (Figure 4.4), suggesting that peptone may stimulate gluconeogenesis independent of *rco-3* and *dgr-1*. Glucose show differentially regulations of NCU09873.1 depending upon the strain background in peptone lacking medium. In the absence of

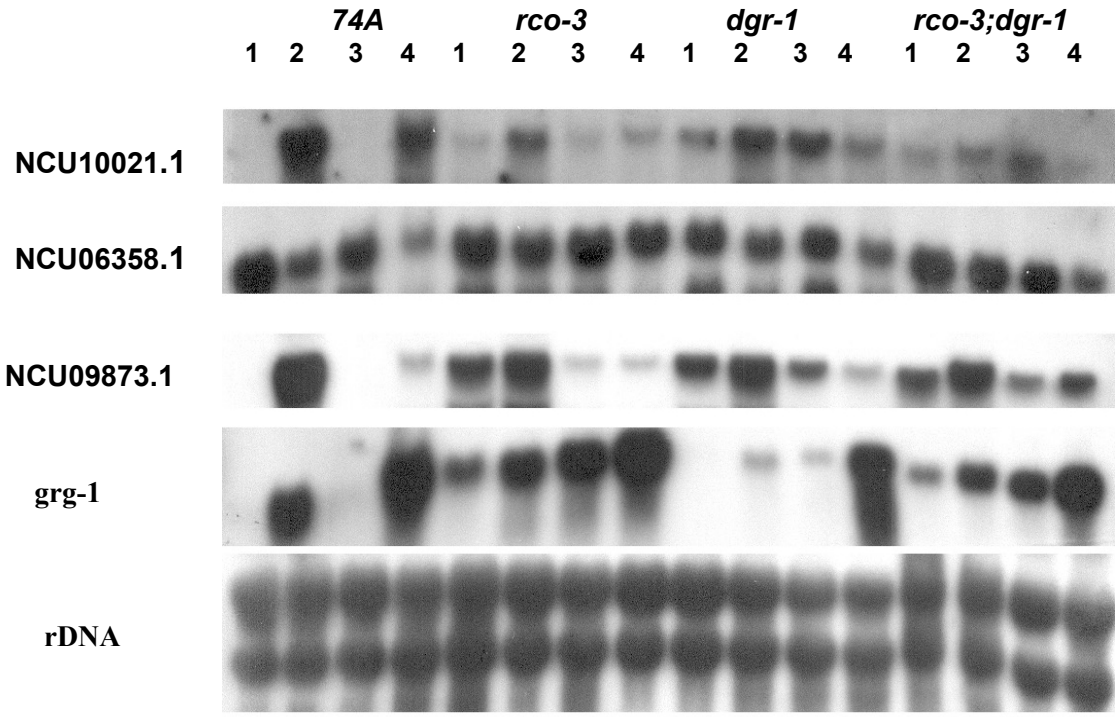


Figure 4.4 Several genes are differentially regulated by glucose, *rco-3, dgr-1* and *rco-3; dgr-1*. Wild type strain (*74A*), *rco-3*, *dgr-1* and *rco-3*; *dgr-1* were grown in liquid minimal medium supplemented with 2% peptone and 2% glucose for approximately 18 hours, mycelium were harvested by filtration and inoculated four different media. 1, minimal medium containing 2% glucose and 2% peptone; 2, minimal medium with 2% peptone but no glucose; 3, minimal medium with 2% glucose but no peptone; 4, minimal medium without peptone and glucose. Genes that are not identified in *N. crassa* are indicated by predicted open reading frame. Genes identified in *N. crassa* are represented by gene name.

peptone, glucose represses NCU09873.1 transcript level in *rco-3*; *dgr-1* double mutant but stimulate it in *dgr-1* and has no effect in *rco-3* (Figure 4.4). Therefore, the expression pattern of *PEPCK* is complicated and depends on strain backgrounds in the absence of peptone. *grg-1* was identified as a glucose repressible gene with unknown function in *N. crassa* (McNally and Free, 1988). *grg-1* mRNA was strongly repressed by glucose in wild type regardless of peptone. In the *rco-3* mutants, there is a much higher basal level of *grg-1* expression in the presence of glucose and glucose limitation further stimulates its expression independent of peptone (Figure 4.4). There is an elevated level of *grg-1* expression in the presence of glucose in the *dgr-1* mutant compare to wild type strain. However, peptone appears to strongly repress *grg-1* expression in the *dgr-1* strain (Figure 4.4). In the absence of peptone, glucose starvation induces *grg-1* expression to a level similar to that of wild type in the *dgr-1* mutant (Figure 4.4). The *rco-3; dgr-1* double mutant has an expression pattern more similar to *rco-3*, indicating that *rco-3* is epistatic to *dgr-1* (Figure 4.4).

Analysis of NCU10021.1

The expression pattern of NCU10021.1 transcripts is similar to the observed regulation of high-affinity glucose transport activity in *N. crassa.* This prompted me to determine whether this gene encodes a high-affinity glucose transporter in *N. crassa*. NCU10021.1 does not define the true coding region for the glucose transporter represented by cDNA clone *SP1A4* because the coding region is truncated at the end of a contig in the *N. crassa* genome sequence. I amplified the entire coding region defined by the sequence of *SP1A4* cDNA clone. This fragment was cloned into an expression vector

(pSM565) containing a ribosomal protein promoter from *M. grisea* to overexpress the transporter gene (pX12) (see materials and methods). Both pSM565 and pX12 plasmids were transformed into wild type strain and RNA was extracted for Northern blot analysis. Northern blots indicate the transformants express the transgene in the presence of glucose (Figure 4.5A). However, the size of the transgene mRNA is slightly smaller than the endogeneous copy of *SP1A4*, which is probably due to the length of the 5' and/or 3' un-translated region. I intended to constitutively overexpress the sugar transport homolog, however, it is clear that most ribosomal protein gene are down-regulated in response to glucose starvation (Chapter II), and the smaller transgene transcript appears to be absent in transformants starved for glucose (Figure 4.5A). To verify the overexpression of NCU100021.1 does not affect glucose regulated gene expression, I examined the expression of NCU09873.1 (*PEPCK*) and NCU06358.1 (sugar transporter homolog), and their regulation was not altered in *SP1A4* overexpression strains (S2, S9 and S13) as I expected (Figure 4.5A). Strain (S2) was selected for further analysis and designated $SP1A4^{OE}$.

In *N. crassa*, duplicated DNA sequences in the genome can be detected and mutated during mating by a process termed RIP (repeat induced point mutation) (Selker and Garrett, 1988; Selker, 1990). RIP mutation generates numerous GC to AT transition mutations and usually results in null alleles. The wild type strain bearing a single ecotopic copy of pX12 (S2) was crossed with the wild type strain. Fifteen random progenies were chosen to examine *SP1A4* transcript level under glucose deprivation

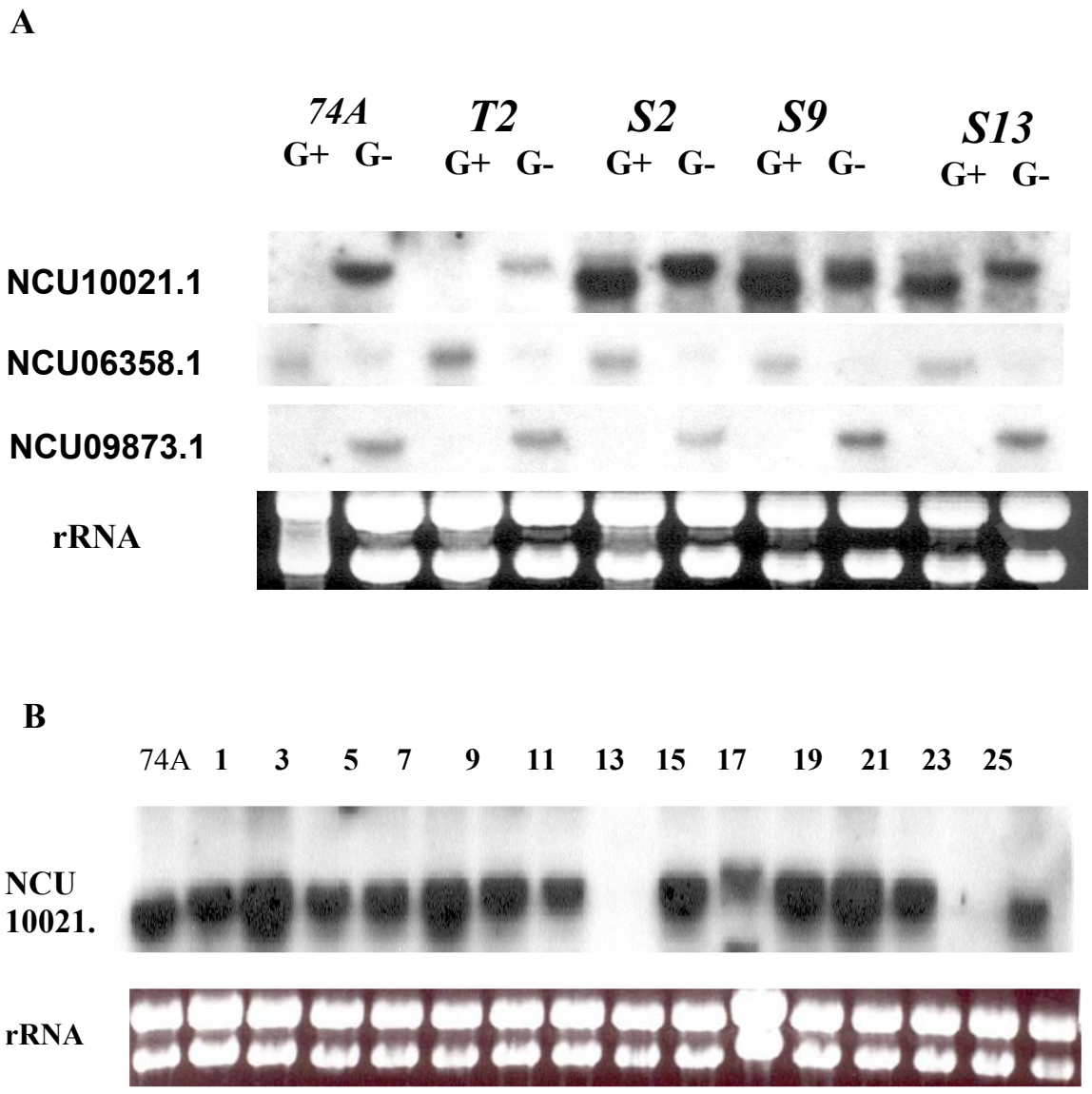


Figure 4.5 Northern blot analyses to examine overexpression and disruption of *SP1A4*. (A) 74A is wild type strain. T2 is 74A transformed with vector lacking *SP1A4* gene. S2, S9, S13 are 74A strains containing NCU10021.1 overexpression constructs. Cells were cultured in minimal medium with 2% glucose and 2% peptone for 18 hours, mycelia were harvested and inoculated into fresh medium with 2% glucose (G+) or without glucose (G-) for two hours. The same membrane was probed with genes (NCU10021.1, NCU06358.1 and NCU09873.1) (B) 1 to 31 are progeny from the NCU10021.1 ripping cross. All strains are grown in minimal medium with 2% glucose and 2% peptone for 18 hours, mycelia were harvested and inoculated into fresh medium lacking glucose for two hours. RNA was extracted to examine NCU10021.1 expression. The rRNA stained with ethidium bromide on the gel is shown as a loading control.

Table 4.4 Glucose and fructose transport in the *74A, SP1A4*^{rip1} and *SP1A4*^{OE} strains.

	Glucose-grown cells			Glucose-starved cells		
	***74A*(r^{2a})**	***SP1A4*^{RIP1}(r^2)**	***SP1A4*^{OE}(r^2)**	***74A*(r^2)**	***SP1A4*^{RIP1}(r^2)**	***SP1A4*^{OE}(r^2)**
Glucose 0.25 mM	**0.25^b (0.99)**	**0.22(0.99)**	**1.4(0.99)**	**17(0.99)**	**6.0(0.99)**	**10(0.99)**
40 mM	**9.5(1)**	**7.4(0.98)**	**14(0.99)**	**25(0.99)**	**20(0.98)**	**19(0.99)**
Fructose 10 mM	**1.1(0.98)**	**0.4(0.99)**	**2.1(0.99)**	**9.2(0.99)**	**13(0.94)**	**9.7(0.99)**

[a] r2 is the correlation coefficient for linear uptake during the time course
[b] Values are nmol glucose transported/min/mg dry mycelia
74A data are taken from previous table for comparison

condition (Figure 4.5B). Two progeny lack *SP1A4* mRNA indicating the endogenous gene had been mutated (Figure 4.5B). Southern hybridization revealed both putative RIP strains contain only the endogenous, mutated copy of *SP1A4* (data not shown). I designated these strains as *SP1A4*^{RIP1} (progeny 15) and *SP1A4*^{RIP2} (progeny 29) for further analysis.

SP1A4 represents a high affinity glucose transporter in N. crassa

I examined glucose and fructose uptake activities in *SP1A4*^{OE} and *SP1A4*^{RIP1} (Table 4.4). If *SP1A4* represents a high affinity glucose transporter, I expect a reduced high affinity glucose transport activity in the *SP1A4*^{RIP1} strain when cells are starved for glucose. I found that high affinity glucose transport in glucose starved cells was significantly reduced (approximately one third of the transport activity of wild type) in the *SP1A4*^{RIP1} mutant (Table 4.4). The *SP1A4*^{RIP2} strain displays similar sugar transport activities as the *SP1A4*^{RIP1} strain (data not shown). In addition, the high affinity glucose transport activity in glucose grown cell should be elevated in the *SP1A4* overexpression strains if *SP1A4* represents a high affinity glucose transporter, which turns out to be the case. I observed a five fold increase of high affinity glucose transport activity in *SP1A4*^{OE} in the presence of glucose compare to wild type strain (Table 4.4). The sugar transport activity in *SP1A4*^{RIP1} and *SP1A4*^{OE} strains suggest *SP1A4* represents a high affinity glucose transporter gene in *N. crassa.*

If the primary role of *SP1A4* is limited to glucose transport, the *SP1A4*^{RIP1} and *SP1A4*^{OE} strains should not affect conidiation and carbon regulation of gene expression in *N. crassa*. In minimal medium containing 2% glucose, *SP1A4*^{RIP1} and *SP1A4*^{OE} grow

primarily as filamentous hyphae as wild type strain (Figure 4.6), which is consistent with *SP1A4*'s role as a glucose transporter. In addition, both *SP1A4*^{rip} and *SP1A4*^{OE} strains are sensitive to sorbose and 2-deoxyglucose as I expected. Furthermore, overexpression of *SP1A4* does not alter the expression pattern of another glucose transporter homolog (NCU06358.1) and a key gene in gluconeogenesis (NCU09873.1) (Figure 4.5A). Taken together, these findings strongly suggest *SP1A4* encodes a simple high affinity sugar transporter (HGT1) (high affinity glucose transporter-1) in *N. crassa*.

DISCUSSION

rco-3 and *dgr-1* display similar phenotypes, strongly suggesting they are in a common regulatory pathway. However, there are some differences between *rco-3* and *dgr-1* phenotypes. Although *dgr-1* and *rco-3* both conidiate in submerged culture, *dgr-1* forms fewer conidiophores than *rco-3* and sorbose resistance is less pronounced. These differences may be due to the less severe glucose transport defects in the *dgr-1* mutant. A major difference between *rco-3* and *dgr-1* is that *ssr* mutations suppress all the *rco-3* phenotypes but only some of the *dgr-1* phenotypes. A simple model to represent these observations is given in Figure 4.7, in which *rco-3* lies upstream of *dgr-1* and controls two pathways regulating glucose transport activities. In this scenario, the *dgr-1* gene lies in only one of the two pathways while *ssr* genes lie upstream of *dgr-1* and can suppress *rco-3* but not *dgr-1*. This model can account for the epistasis of *rco-3* over *dgr-1* but does not explain the observed suppression of sorbose resistance in *dgr-1* mutants by the

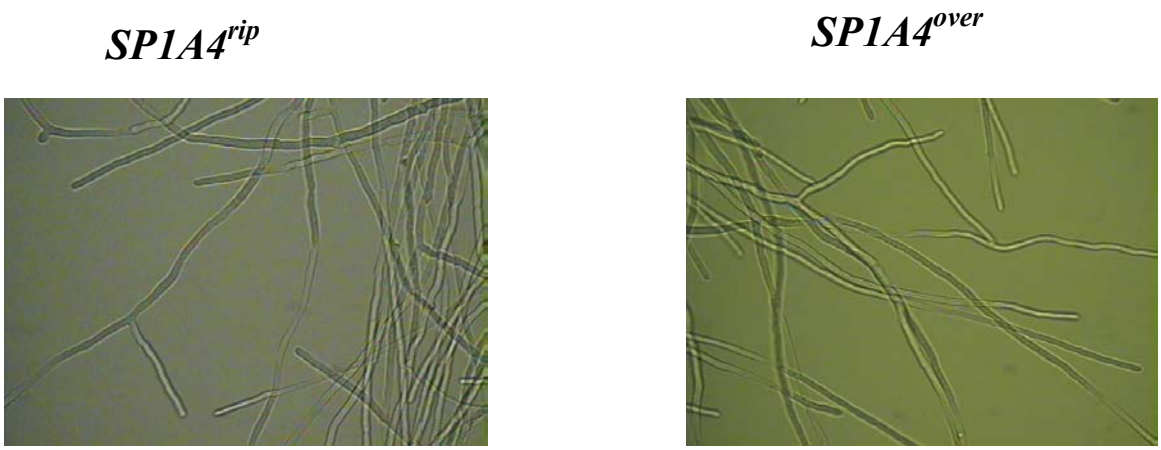


Figure 4.6 Conidiation of *SP1A4*^{rip} and *SP1A4*^{over} in liquid medium. *SP1A4*^{rip} and *SP1A4*^{over} grow primarily as filamentous hyphae in minimum medium with 2% glucose.

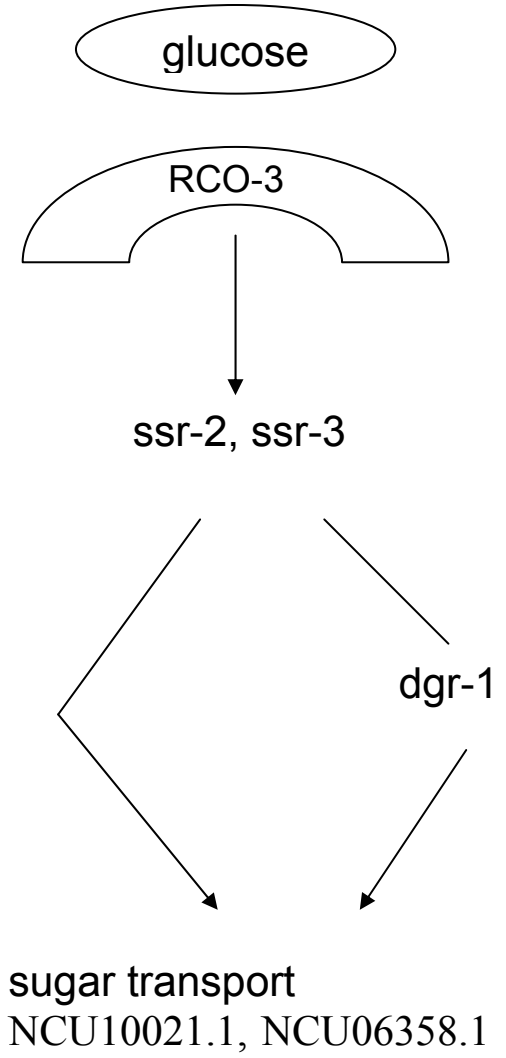


Figure 4.7 Model of the *rco-3* gene regulating sugar transport in *N. crassa*.

ssr genes. However, recent transcriptional profiling experiments with *rco-3* and *dgr-1* mutants show remarkably similarity in the expression patterns for the two mutants (Chapter III). Therefore I favor a model incorporating both genes as part of a common regulatory circuit.

rco-3 was proposed to be the counterpart of *snf3* and *rgt2* (two glucose sensors of *S. cerevisiae*) (Madi *et al.,* 1997). snf3 and rgt2 regulate hexose transporter gene expression, while *rco-3* also regulates several sugar transport homologs in *N. crassa*. One of these homologs (HGT1, High-affinity Glucose Transporter) has an expression pattern similar to the measured high affinity glucose transport activity. My analysis suggests that HGT1 does encode such an activity. Several sugar transporter homologs are regulated in a manner expected for glucose or fructose transporters and functional analysis of these genes should be useful in identifying other glucose transporters and in the generation of mutant strains that have greatly reduced glucose transport activity. Such strains have proved very useful in elucidation of glucose transporter function and mechanisms of glucose regulation in *S. cerevisiae* (Özcan *et al.,* 1998; Rolland *et al.,* 2001). Although heterologous expression of *hgt1* mRNA in *N. crassa* (*SP1A4*^{OE}) glucose grown cells was achieved, and there was approximately a four-fold increase in high affinity glucose transport activity observed (Table 4.4), the increased rate of glucose uptake was marginal compared to cells derepressed for high-affinity glucose uptake. One possibility is that post-translational regulation of *hgt1* transporter in the presence of glucose may occur to inactivate HGT1, as has been observed for maltose transporters in *S. cerevisiae* (Brondi *et al.,* 2001**).** Also, there are likely to be several high affinity

glucose transporter genes in *N. crassa* based on the analysis of glucose transport activity in the *hgt1* mutant and from the correlation of the patterns of sugar transporter homolog gene expression and sugar transport activity. In addition, the *N. crassa* genome sequence revealed that there are more than thirty hexose transporter homologs present in the genome, representing one of the largest gene families in *N. crassa* (Galagan *et al.,* 2003). Therefore, *hgt1* very likely represents just one of several sugar transporters that contribute to high affinity glucose transport in *N. crassa*.

rco-3 was proposed to encode a sugar sensor in *N. crassa* based on the indirect evidence of its phenotype and its homology to sugar transporters (Madi *et al.,* 1997). Part of the evidence supporting the view that *snf3* and *rgt2* were sugar sensors is their role in regulating the expression of other hexose transporter genes in *S. cerevisiae*. Here I have identified a high affinity glucose transporter gene and its expression is under the control of *rco-3*. In addition, the *rco-3* mutants display altered transcriptional regulation of several other sugar transporter homologs (Figure 4.4) (Chapter III), which may encode high or low affinity glucose transporters. In contrast, overexpression the *hgt1* gene does not alter the regulation of several glucose regulated genes including a glucose transporter homolog (Figure 4.5). *rco-3* has altered transport activities in three independent sugar transport systems. It is unlikely that *rco-3* functions simply as a sugar transporter that functions in three different transport systems. *rco-3* induces conidiation in submerged culture without nutrient limitation which could be due to the sugar transport defect, however, disruption of *hgt1* does not affect asexual development. Therefore, disruption of a putative sugar transporter in general does alter the ability to

repress conidiation. Furthermore, transcript profiling demonstrates that *rco-3* regulates a wide variety of genes that are not regulated in response to glucose starvation (Chapter III). This observation demonstrates that at least part of the effect of *rco-3* on gene regulation is independent of glucose limitation. Finally, the suppressors of *rco-3* restore sugar transport regulation in *rco-3* but not *dgr-1* mutants suggesting that the suppressors must be part of a regulatory pathway involving *rco-3* and not simply mutations that constitutively elevate sugar transporter expression. Taken together, I conclude that *rco-3* functions as a sugar sensor and not as of sugar transporter in *N. crassa*.

CHAPTER V

CONCLUSIONS

Carbon availability is important for cell differentiation in many microorganisms. In the filamentous fungus, *Neurospora crassa*, macroconidiation can be induced by nutrient limitation in submerged culture. Glucose is a primary carbon source for *N. crassa*. Therefore, understanding glucose metabolism and glucose regulation of gene expression will help to elucidate how nutrient signals trigger cellular differentiation in *N. crassa*.

The *rco-3* mutant was isolated based on its elevated expression of a conidiation specific gene (*con-10*) (Madi *et al.,* 1994). Subsequent cloning and characterization indicate that the *rco-3* gene is a regulator of sugar transport and conidiation in *N. crassa*. A previously isolated 2-deoxyglucose resistant mutant (*dgr-1*) phenotypically resembles *rco-3* and *rco-3* is allelic to another 2-deoxyglucose resistant mutant (*dgr-3*). A suppressor approach was used to identify genes that may define components of a regulatory pathway controlling sugar transport and development. Three distinct suppressors that suppress the sorbose resistance phenotype of *rco-3* mutants were identified and designated as *ssr-1*, *ssr-2* and *ssr-3*. All suppressors remedy the sorbose resistance, derepression of conidiation and the induction defect in sugar uptake in the *rco-3* mutants. *dgr-1* displays a similar but not identical phenotype as *rco-3*, therefore *dgr-1* may be involved in the *rco-3* regulatory circuits. Characterization of *rco-3*; *dgr-1*, *ssr-2*; *dgr-1* and *ssr-3*; *dgr-1* double mutants suggest that *rco-3* is epistatic to *dgr-1*.

rco-3 was proposed to function as a sugar sensor (Madi *et al.,* 1997). Thus, understanding sugar catabolism and carbon regulation in *N. crassa* will provide a basis to further elucidate the *rco-3* signaling pathways. Therefore, I helped to generate a cDNA microarray containing 1363 unique *N. crassa* genes. An analysis of the transcriptional profiling of *N. crassa* in response to glucose availability revealed that 17% of the genes on the microarray were differentially expressed. The major transcriptional response was observed for genes belonging to central metabolism and growth. Northern blot analysis for some of these genes and previously published measurements of gene expression and enzyme activities were found to generally agree with microarray data. General biosynthesis and glucose utilization was repressed while utilization of alternative carbon or existing nutrients were stimulated upon glucose deprivation. Comparing *N. crassa* profiling data with the published diauxic shift data from *S. cerevisiae* (DeRisi *et al.,* 1997), revealed that *S. cerevisiae* and *N. crassa* share a similar, but not identical, transcriptional response pattern for genes belonging to central carbon catabolism. The studies indicate that *N. crassa* simultaneously exploits glucose through fermentation and respiration in aerobic cultures, a finding that is consistent with previous measurements of ethanol production in *N. crassa*. This finding may relate to the ecological niche occupied by *N. crassa* and provides useful information for potential metabolic engineering of *N. crassa* for bioethanol production from plant substrates.

To further assess the relationship between *rco-3* and *dgr-1* and define the function of these two genes, I examined transcriptional responses to glucose status in *rco-3* and *dgr-1* mutants via microarray technology. *rco-3* and *dgr-1* display similar

expression patterns for the majority of genes on the microarray supporting a close functional relationship between them. My results indicate that *rco-3* and *dgr-1* induce conidiation at least partially independently of sugar transport defects. The observation that genes involved in the TCA cycle, the glyoxylate cycle and gluconeogenesis have lost glucose regulation in *rco-3* and *dgr-1* mutants indicates that *rco-3* and *dgr-1* regulation is not due strictly to glucose deprivation. This finding supports the model that *rco-3* and *dgr-1* control multiple pathways to regulate different aspects of cellular physiology. The differential expression pattern of multiple components involved in different signaling pathways, such as MAP kinase, adenylate cyclase, protein kinase, protein phosphatase and a variety of transcription factors further support this view. In addition, I have identified a high affinity glucose transporter gene in *N. crassa*, whose transcription is under the control of glucose, *rco-3* and *dgr-1*.

Microarray technology is a recent addition to functional genomics tools available in *N. crassa* and allowed me to study the expression pattern of 1363 genes in one hybridization experiment, whereas Northern blot analysis examines only expression of one gene at one time. However, validation of microarray data is a very important step in interpretation. Variation between microarray experiments may reflect defects in microarray spotting, uneven background levels, or imperfect replication of probe synthesis between experiments. Most of my microarray data are consistent with Northern blot analyses performed by myself and previously published Northern blot data and enzyme activity measurements (Chapter II and Chapter III). However, I do find some apparent differences between microarray and Northern blot data and these

differences could arise for several reasons. First, although I used several strategies to reduce variation in microarray hybridizations, such as combining RNA isolated from multiple cultures, replicate microarray hybridization including dye-swap hybridization, and using statistical measurements to filter out statistically questionable data points, there is still variation that exists in microarray analysis. The data present in this report are the averaged values of at least three replicate hybridizations for each test condition. I can not rule out the possibility, that any particular value from a pair of spots on the microarray is an outlier value in one hybridization experiment that might significantly influence the average value for that EST. This would require manual examination of each data point, however, computer programs could be written to speed this process by automating examination of the standard deviations of each set and determining if additional filtering of individual data points is necessary. Second, microarry technology examines a large number of genes at the same time; the scanning setting to quantitate hybridization is adjusted based on the signal intensity of the majority of genes on the microarray. Therefore, some highly expressed genes may be saturated and the measured values may be out of the linear range of the detector, while the exposure time in Northern blot analysis can be optimized for each individual gene. Third, I used combined RNA preparations for microarray hybridization experiments while Northern blots used RNA from just one of the growth experiments. Thus, the difference between Northern blot and microarray results may reflect true variation occurring between growth experiments. In addition, it is also known that there is approximately 10% error in EST assignment based on sequencing a sample of the clones on the microarray (Lewis

et al., 2002). I used PCR products from the sequence verified EST clones as probes for Northern blot analysis, however, not all EST clones on the microarray have been verified.

Differences between microarray and Northern blot results indicate that one of the problems discussed above may exist for certain individual gene. However, much of my array analysis is based on the expression pattern of genes representing an entire pathway instead of one particular gene. Therefore, checking of every gene in glycolysis, the glyoxylate cycle, or the TCA cycle by Northern blots is not critical for a general view of metabolism. However, Northern blot analysis for certain individual genes of interest, such as the Hsl7 homolog and adenylate cyclase (Chapter III), is warranted. I also found that in a few cases with multiply represented genes (different ESTs spotted for the same gene), different EST clones displayed inconsistent results in the microarray experiments. This discrepancy could be resolved by sequencing EST clones (if some of these are among the 10% of clones with incorrect gene assignments) and Northern blot analysis (if some of these are unevenly spotted on the microarray slide).

In summary, my studies identified several new components and a variety of target genes of the putative *rco-3* signaling cascade. The phenotypic characterization and transcriptional profiling of *rco-3* and *dgr-1* strains as well as suppressor analysis indicate that *rco-3* and *dgr-1* are part of a common regulatory circuit. All the data present in this report, including suppressor analysis, transcriptional profiling and cloning of a putative glucose transporter, demonstrates that *rco-3* functions as a sugar sensor in *N. crassa*.

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Publications

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Xin Xie, Alejandro Correa, Zach Lewis, Deborah Bell-Pedersen and Daniel J. Ebbole (2003) Global analysis of the response of *Neurospora crassa* to glucose starvation.

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